Role of Physico-chemical Environmental Factors in the Emergence and Development of Insecticides Resistant Mosquito in Nigeria.

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By

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Dedication

This work is dedicated to the memory of my beloved maternal grandmother. Death denied us the pleasure of celebrating this achievement together, but I will always be grateful to you for the culture of hard work, dedication and discipline you instilled in me as a child.

Acknowledgments

Sincere gratitude to my supervisory team; Dr Yusuf Deeni, Prof. Graeme Walker, Dr. Andrew Spiers and Dr. Simona. Hapca for their relentless effort in making my PhD dream a reality. Also, my sincere gratitude to Nigerian Petroleum Technology Development Fund (PTDF) for funding this PhD study. Long live Nigeria.

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Symbols and Abbreviations

α	Alpha
β	Beta
β-NF	Beta-naphtho flavone
γ	Gama
μ	Micro
ε	Extinction coefficient
°C	Degree Celsius
AChE	Acetylcholinesterase
ADP	Adenosine diphosphate
AFRO	African regional office
AhR	Aryl hydrocarbon receptor
AhRNt	Aryl hydrocarbon receptor nuclear translocator
AIDs	Acquired immunodeficiency diseases
An.	Anopheles
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BHC	Benzene hexachloride
BOD	Biological oxygen demand
CAR	Constitutive androstone receptor
CDNB	Chlorodinitrobenzene
CYT	Cytochrome
DDE	Dichlorophenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
Di	Dieldrin
DNA	Deoxyribonucleic acid

DO	Dissolve oxygen
DTNB	5' 5'-Dithio[2-nitrobenzoic acid]
EcRE	Ecdysone response elements
EMRO	Eastern Mediterranean regional office
EURO	European regional office
FAO	Food and agricultural organisation
GCL	Glutamate cystein ligase
GCLC	Glutamate cystein ligase catalytic subunit
GCLM	Glutamate cystein ligase modifier subunit
GS	Glutathione synthase
GST	Glutathione S-transferase
HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
IRS	Insecticides residual spray
ITNs	Insecticides treated nets
kdr	Knock down resistance
KDA	Kilodalton
LLITNs	Long lasting insecticides treated nets
mRNA	Messenger RNA
1-NA	1-Naphthyl acetate
2-NA	2-Naphthyl acetate
NAC	Nacetyl cystein
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
$NADP^+$	Nicotinamide dinucleotide phosphate oxidized
NAPQI	N-acetyl para-benzoquinoneimine
UNICEF	United nations children educational fund

OP	Organophosphates
PC	Principal components
PCB	Polychlorobiphenyls
PCR	Polymerase chain reaction
Pi	Inorganic phosphate
QTL	Quantitative trait loci
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SEARO	South east Asian regional office
TDS	Total dissolved solids
TNB	5'-Thionitrobenzoic acid
tRNA	Transfer RNA
WHO	World health organisation
XREs	Xenobiotic response elements

Declaration

I hereby declare that the following thesis is based on the results of investigations conducted by myself, and that it is of my own composition. This thesis has not, in whole or in part, been previously presented for a higher degree or qualification. Work other than my own is clearly indicated in the text by reference to the relevant researchers or publications.

Abdullahi A Imam

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Abstract

The aim of this study is to investigate the role of physico-chemical environmental factors present in mosquito breeding ecologies as sources of selection pressure for the emergence and development of insecticides resistance in *Anopheles gambiae* from Northern Nigeria. Prospecting for, and sampling of *An. gambiae* larvae was carried out from many breeding sites in towns and villages spread out across Kano and Jigawa states of Northern Nigeria. The breeding sites visited and sampled were grouped into three study zones according to the type of human related activities taking place around the mosquito breeding environments. Larval densities (per litre of breeding water) and water chemistry analysis were carried out from all the breeding sites visited. Detoxification enzymes; Cytochrome P450, Glutathione S-transferases and Esterases as well as glutathione assays were carried out on the three life stages of the sampled *An. gambiae* using the relevant assay protocols. The results showed that the levels of the physical environmental factors; pH, temperature, conductivity, transparency, dissolved oxygen and biological oxygen demand did not vary significantly ($p<0.05$) across the three studied zones. The levels of the chemical environmental factors; total dissolved solids, sulphate, phosphate, nitrite and nitrate ions were significantly (p0.05) higher in study zone A compare to the other two zones, while carbon content and oil and grease were significantly ($p<0.05$) distributed in study C than the other two zones. Likewise, the activity of P450s was higher in study zone C while those of GST and CEs were significantly ($p<0.05$) higher in study zone A compared to the other two zones. Additionally, most of the chemical environmental factors were significantly ($p<0.05$) associated with larval density and activities of the detoxification enzymes. Oxidized glutathione (GSSG) levels were also highly associated ($p<0.05$) with the activities of the three enzymes studies. A deduced model of multivariate regression established all the chemical environmental factors and some of the physical environmental factors as producing a combined effect on larval density and detoxification enzymes activities. These observations showed that *An. gambae* emerging from breeding sites located in study zones A & C may have, through adaptive tolerance, acquired the necessary detoxification machinery that could allow it to rapidly develop resistance to various classes of insecticides. Finally, the contribution and implications of the findings in this study to the challenges facing vector control as a tool in malaria management, specifically, the contribution to the underlying mechanism of insecticides resistance and the various factors selecting for resistance development in *An. gambiae* were discussed.

CHAPTER 1 GENERAL INTRODUCTION

1.1 History and Epidemiology of Malaria

"While keeping in mind the realities one can nevertheless be confident that malaria is well on its way towards oblivion. Already as a malariologist, I feel premonitory twinges of lonesomeness and in my own organisation I am now a sort of last 'survivor'. So perhaps it is fitting that I should take this backward glance at the fascinating pages of malaria history." This quotation was extracted from Paul Russell's preface of 'Man's Mastery of Malaria' published in 1955 (Russell, 1955). With the present state of malaria situation in the world, this extract now seems astonishing. Scientists all over the world have become less and less optimistic about the prospects for global malaria control since the last 50 years when the series of lectures and seminars culminating in Paul Russell's paper was given.

The parasitic association between the human race and the malaria parasite has had a long evolutionary history (Wiesenfeld, 1967; Coluzzi, 1999; Joy, Feng and Mu *et al.*, 2003). The hypotheses inferred from changes in human ecology largely supported by advances in bioinformatics (Hume, Lyons and Day, 2003; Hartl, 2004) that populations of *Plasmodium falciparum* increased about 10,000 years ago in African continent and spread all over the world. This rapid spread of malaria parasite was thought to coincide with growth in the human population facilitated by the dawn of agriculture (De Zulueta, 1987; De Zulueta, 1994). However, an earlier smaller wave of migration during the Pleiotoscene period was thought to have preceded this expansion (Joy, Feng and Mu 2003).

According to the world Health Organisation (WHO), about half of the world population (3.3 billion) is at risk of malaria. More than 216 million cases were reported in 2010 alone, with over 660, 000 deaths recorded (WHO, 2013). Over 300 million people are infected yearly, mostly in areas of poverty and low economic growth (Sachs and Malaney, 2002). According

to the World Health Organisation, about 109 countries were endemic for malaria in 2008, out of which 45 are found within the WHO African region (WHO, 2013). Sub-Saharan Africa accounts for almost 90 percent of all malaria cases and deaths attributed to malaria alone stands at about 20 percent (WHO2013). The disease is implicated in widespread sufferings and premature death, imposes financial hardships on poor households and retard economic growth and development as well as decreasing living standard. The management of malaria has also been hampered by the development of resistance to the two major drugs used for its treatment; first chloroquine and later sulfadoxine-pyremethamine (Phyo *et al.*, 2012). While many drugs have been and are still being developed to combat malaria, vector control by recourse to environmental management, educational programmes and the use of chemical and biological agents still remain the most effective alternative to reducing the risk of malaria parasite transmission (WHO, 2006). Out of these alternatives, the use of chemicals in the form of insecticides and insecticide treated bed nets is probably the best available option for vector control in sub- Saharan Africa and other less developed parts of the world. With the evolution of resistance to insecticides, this too is facing serious challenges. It has been estimated that management of malaria alone costs the African countries about 12 billion dollars yearly. This has lead to considerable retardation in economic growth and development (Sachs and Malaney 2002).

The rapid global human population growth in the 20th century from approximately 1 to 6 billion, present an important demographic implications for the percentage of people exposed to the risk of malaria attack. At the turn of the century, the global population at risk of malaria has decreased from 77 percent to 46 percent in 1994, increasing to 48 percent again in 2002 due to increased population in an unchanged or fixed geographic distribution. In absolute terms therefore, the number of population at risk has increased consistently from 0.9 to 3 billion from 1900 to 2002. Therefore at the turn of the 21st century, estimates put the risk

population at 48 percent, a situation that has deteriorated since then (WHO, 1999; Triggand and Kondrachine, 1998).

1.1.1 Malaria Management and Control

Great improvement in the management and control of communicable diseases was realised during the 19th century principally due to advances in environmental management (Omran, 1971; Mackenbach, 1994; Curtin, 1989; Doll, 1992; Amstrong, Conn and Pinner, 1999). A parallel improvement in social conditions (particularly housing) as well as changing land use (particularly agricultural practices) also contributed significantly to the global reduction or decrease in the distribution of malaria (De zulueta, 1994; Bradley, 1992; Bruce-Chwatt and De zulueta, 1980; Kitron, 1987; Dobson, 1997). Improved social and economic conditions which were also rampant during this era were coincidental with the gains in malaria control and these two forces, though spatially separated, have remained undiminished throughout the 20th century. According to analyses, malaria is not an obligate tropical disease, but more precisely, one that has been progressively restricted to the tropical regions in the 20th century as a result of development and control (Russell, 1955; Coluzzi, 1999; De zulueta, 1994; Haworth, 1988; Onori, Beales and Gilles, 2002). This global reduction and the resultant restriction to the tropical region followed several distinct stages.

The first stage referred to as the 'sanitation era' of malaria control focused primarily on environmental management and control of mosquitoes breeding sites (Russell, 1955; Covell, 1941; Bradley, 1966; Gilles and Lucas, 1998; Konradsen, Vander Hoek , Amerasinghe, Mutero and Boelee, 2004). The discovery by Ross (Ross, 1923; Nye and Gibson, 1997; Bynum, 1999) of the importance of the *Anophelene* mosquitoes in the pathogenesis of avian malaria in 1898 and the subsequent de nonstration by Grassi of the full transmission cycle of the human malaria later that year (Sharman, 1998; Fantini, 1999; Dobson, 1999) as well as

the success recorded in mosquito control in the Panama canal (Gorgas, 1915; Simmons, 1939; McCullough, 1977), Indonesia (Swellengrebel, 1950), Malaysia (Watson, 1921), the mines and plantation of the Zambian Copper belt (Watson, 1921; Utzinger, Tozan, Dounami and Singer, 2002) and *An. gambiae* eradication in Brazil (Soper and Wilson, 1943; Killean, Fillinger, Kicle, Gouagna and Knols, 2002) and Egypt (Killean *et al.*, 2002) lend credence to such approaches despite obvious failures in Sardinia (Logan, 1953), Sierra Leone and India (Russell, 1955; Ross, 1923). The presence of commercial incentives for investment and logistic feasibility was judged to be hugely responsible for the adoption of species sanitation approach in those areas where it was adjudged successful (Bradley, 1966; Bruce-Chwatt and De zulueta, 1980 De zulueta, 1998).

Large-scale wide-area approaches to malaria management and control was first introduced in the 1940s following the discovery of the residual insecticidal properties of dichlorodiphenyltichloroethane (DDT) and its effect on those engaged in malaria interventions should not be underestimated. The World Health Organisation recognising the enormous importance of this approach endorsed it through the global malaria eradication programmes from 1955 to 1969 (Litsios, 2002). This global approach essentially involved the widespread use of DDT to interrupt transmission in an 'attack phase' and chemoprophylaxis to eradicate malaria in the later 'consolidation phase' of intervention (Mc Donald, 1965; Litsios, 1966; Pampana, 1969; Beales and Gilles, 2002; Spieldman and D'Antonio, 2001). The problems, successes and long term effects of these phases of malaria intervention has been well documented (De zulueta, 1998; Kitron and spieldman, 1989) along with the subsequent resurgent of malaria in India (Sharma, 1996) and the complete disregard for Sub Saharan Africa in the global eradication effort (Litsios, 1966). The decrease in the global malaria distribution between 1946 and 1965 largely coincides with this eradication era.

The control intervention of malaria in the attack phase culminating in the aftermath of the discovery of the residual insecticide properties of DDT was essentially achieved through two main vector control initiatives: indoor (house) residual insecticide spraying (IRS) and insecticides treated mosquito nets (ITNs). A comprehensive study has been carried out on the health effects of ITNs in two Cochrane Reviews; one for the general population and one for pregnant women (Lengeler, 2004; Gamble, 2006). Malaria control using indoor residual spraying has a long and distinguished history. It can be claimed without any doubt that using DDT mainly, malaria was eliminated or greatly reduced in Europe, Russia, Asia and Latin America (Schiff, 2002; Lengeler, 2003; Roberts, 2004). IRS still forms a major avenue of malaria control intervention in many parts of the world. A historical review of the contribution of IRS investigated before and after the control interventions in Southern Africa reveals that IRS continues to protect some 13 million people in the entire Southern African sub-region, including Zimbabwe, Swaziland, Namibia, South Africa and Mozambique (Mabaso, 2004). Spectacular reductions in malaria parameters and vector densities were recorded after the implementation of control interventions, and even in some instances, total elimination of vectors was achieved. Another study reviewed the health implication of 36 IRS programmes in 19 sub-Saharan African countries where comparative analyses of parasites biting rates, and other malariological outcomes was carried out before and after the control operations in each of the five major eco-epidemiological zones (Kouznetsov, 1977). A major shortcoming of most of these studies is that they simply documented time trends of malaria parameters with no appropriate control groups. Most of the recent programmes on impact assessment are also characterised by this shortcoming (Sharp, 2002; Tseng, 2008; Teklehaimanot, 2009; Kleinschmidt, 2009). Hence while there is no doubt that IRS has contributed immensely to malaria reduction and improved health outcomes worldwide,

assessments up to the present day do not allow for a definite quantification of the health and environmental implication.

IRS is thought to operate in two ways; by repelling mosquitoes from entering houses and by killing female mosquitoes that are resting inside houses after having taken a blood meal. This implies that IRS is most effective against endophilic mosquitoes i.e. mosquitoes resting indoors. On the contrary, ITNs show a greater degree of personal protection compared to IRS which relies largely on a vectorial mass effect: the increased mortality of adult mosquitoes mostly following feeding leads to a reduction in transmission. However, IRS has the advantage of employing the use of a wide range of insecticide products unlike ITNs which is mostly restricted to the pyrethroid class of insecticides. The various types of insecticide products recommended by the World Health Organisation for use in indoor residual spraying include but not limited to DDT wettable powder (WP), malathion WP, fenitrothion WP, bendiocard WP, propoxur WP, alpha-cypermethrin WP (WHOPES, 2007). This extended wide range of insecticide products has the important advantage of guiding against the development of insecticide resistance and hence the long term sustainability of vector controls interventions. The potential adverse health effects of insecticides used for IRS, especially DDT, constitute an important issue worthy of serious attention, but one that is beyond the scope of this review. However, to mention but just a few, two studies had earlier outline the cost and health implication of IRS (Curtis, 2001; Lengeler, 2003) including a comparative analyses of effects of IRS against that of ITNs but neither of them was conducted systematically or assessed the methodological quality of the included studies. A comprehensive and standardised cost and cost-effective analyses for the ITNs distribution models and for the two IRS programmes in South Africa was presented by Yukich, (2008).

We have witnessed and still witnessing a substantial expansion in malaria control programmes employing the use of insecticides especially and quite recently in Nigeria and

other parts of sub Saharan Africa , the extensive and rapid rolling out of long lasting insecticides treated bed nets (LLITNs) together with the already available indoor residual spraying (Roberts and Enserink, 2007). As pointed out earlier, about 12 insecticide products have been approved by the World Health Organisation for IRS, all of which belong to only three chemical classes of insecticides (organochlorines, organophosphates, and carbamates). All of these three classes are nerve poisons which either target acetylcholinesterase in the synapses or the voltage gated sodium channel on the insect neurones. The chemical arsenal is even more limited for impregnated insecticides bed nets confined to only six insecticide products all of which belong to pyrethroid class of insecticide. These same classes of insecticides are also widely applied to the control and management of agricultural pest in Africa and this can pose an additional selection pressure on mosquitoes when insecticide contaminated ground or surface water permeates their larval breeding sites. The result of this intensive exposure to insecticides is the inevitable development and evolution of resistance to insecticides in *Anopheles* mosquitoes that vector malaria parasites. The first report of resistance to organochlorines; DDT and the now obsolete dieldrin, in African malaria vectors was reported in the 1950s and 1960s (Brown, 1958). Resistance to pyrethroids was detected in African mosquitoes much later in 1993. Since then there have been published reports of pyrethroid resistance in various species of *Anopheles* mosquito population in countries from east, west, south and central Africa (Ndyemai *et al.*, 2009; Munlenga *et al.*, 2008 and Awolola *et al.*, 2009; Protopopoff *et al.*, 2008; Hargreaves *et al.*, 2000 and Okoye *et al.*, 2008). Recently, populations of *An. gambiae* resistant to organophosphate and carbamate were reported in West Africa (Corbel *et al.*, 2007).

1.2 Mosquito

Mosquito is a Spanish or Portuguese word meaning little fly. It belongs to the insect family of *culicidae* (from Latin word 'culex' meaning midget or gnat). The scientific classification of mosquito is given below:

Kingdom*:* *Animalia*

Phylum*:* *Arthropoda*

Class: *Insecta*

Order: *Diptera*

Suborder*:* *Nematocera*

Intraorder: *Culicomorpha*

Superfamily*:* *Culicoidea*

Family: *Culicidae*

Subfamily: *Anophele, Culicinae, Toxorlynchitinae*

(Harbach, 2008). Mosquito share structural resemblance with two other members of the family; crane flies (family: *Tipulidae*) and chironomid flies (Family: *Chironomides*), with which they are sometimes confused by a casual observer.

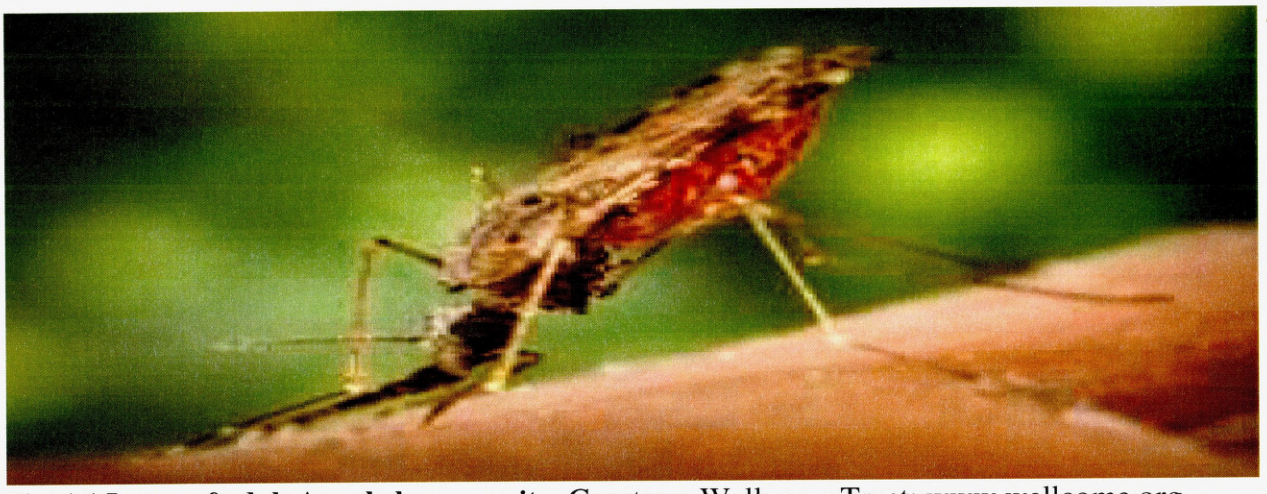

Fig. 1.1 Image of adult Anopheles mosquito. Courtesy, Wellcome Trust; www.wellcome.org

Species of mosquito numbers about 3500, and are distributed all over the world. The female members of these species feed on human blood and are therefore vectors of a number of communicable diseases affecting millions of people annually (Afshin, 2003). The life cycle of mosquito consist of four stages; egg, larva, pupa, and adult also referred to as imago. The adult female mosquitoes lay their eggs in standing water bodies such as lakes, salt marsh, water puddles, a natural reservoir, or on a plant or artificial water container. The larva has a well defined head with mouth brushes used for feeding, no legs, a thorax and a segmented abdomen. The larvae use spiracles located on their eight abdominal segments to breathe. They can also use siphon to obtain air and therefore must come to the surface frequently. Microorganisms such as algae and bacteria found on the surface micro layer constitute the major food for the larvae. They mostly live at or near the surface and dive below only when disturbed. The pupa commonly referred to as 'tumbler' is comma shaped with the head and thorax merged into a cephalothorax with the abdomen. The pupa also floats just underneath the water surface. The pupae like the larvae also come to the surface to breathe with a pair of respiratory trumpets located on the cephalothorax. The pupa does not feed during its stage of development, and few days after the formation of the pupa, it rises to the surface of the water, the dorsal surface of the cdephalothorax splits and the adult mosquito emerges. The pupal stage has less activity compared to the larval stage.

The time taken for the egg to develop into adult mosquito varies among species and is influenced by factors such as ambient temperature. In some mosquito species the duration from egg to adult can be as short as 5 days while in some it take up to 40 days especially in tropical conditions. The mating between adult mosquitoes takes place few days after they emerged from the pupal stage. In most of the species, mating takes place when the male mosquitoes forms large swarms usually around dusk and the females fly into the swarms to mate. Both the male and the female adult mosquitoes feed on nectar or other sources of sugar but the female in addition to nectar also obtain blood meal from a host. After obtaining blood meal, the female rest for a few days during which the blood is digested and eggs developed. In most species this resting period takes two to three days in tropical conditions. After laying their eggs, the female resume host seeking. This cycle of feeding and resting continues until the mosquito dies. The male live for a week while most female lives for several weeks (2-3) at most in nature, but can live longer in captivity. How long they live usually depend on temperature, humidity, as well as their ability to successfully obtain a blood meal while avoiding host defences.

1.2.1 Mosquito and Malaria

Ronald Ross in 1895 embarked on a quest to determine whether mosquitoes vector malaria parasites of humans. For two years in Sekundeland, India, his studies revolved around observations of what is now believed to be insusceptible mosquito species. Although McCullum in 1897 revealed the true nature of flagellation of plasmodium in the blood meal of insect mosquitoes, the preliminary observation leading to this conclusion was started by Ronald Ross (McCullum, 1897). Ross grew 20 adult brown mosquitoes from larvae collected in July 1897, and after a volunteer infected with crescents of malignant tertian malaria was found, he embarked on a four day study which involved infecting the prepared mosquitoes with the blood of the malaria infected volunteer (Ross, 1897). This study, published in British

Medical Journal in 1897, marked the beginning of the most influential stories for malaria research and control (Sinden, 2007)

Although the relationship between man and malaria is that of parasitism, it is the existence of another parasitic relationship; that between mosquito and man, that is responsible for the spread of malaria. Four major species of *plasmodium* are capable of transmitting malaria; *plasmodium falciparum, plasmodium vivax, plasmodium ovale and plasmodium malariae.* These four species are vectored by various species of *Anopheles* mosquitoes, the most common one being *Anopheles gambiae*. Only the female species of mosquitoes actually feed on blood meal which they use to nurture their eggs. The mosquitoes infect man with *plasmodium* in the course of taking this blood meal. The life cycle of the *plasmodium* in humans start from the liver where they multiply inside the hepatic cells, turning into merozoites which then enter into red blood cells. The cycle repeats itself when an uninfected female mosquito bites an infected human and takes up blood meal containing the parasites which they transfer into another uninfected human and the cycle continues. The elimination of either the mosquito or the *plasmodium* parasites would seem like a simple solution (CDC, 2006; WHO, 2006).). Below (Fig. 1.2) shows the life cycle of the major malaria parasites; *Plasmodium falciparum*, P*lasmodium vivax, plasmodium ovale* and *Plasmodium malariae* between human host and malaria vector (female Anopheles mosquito).

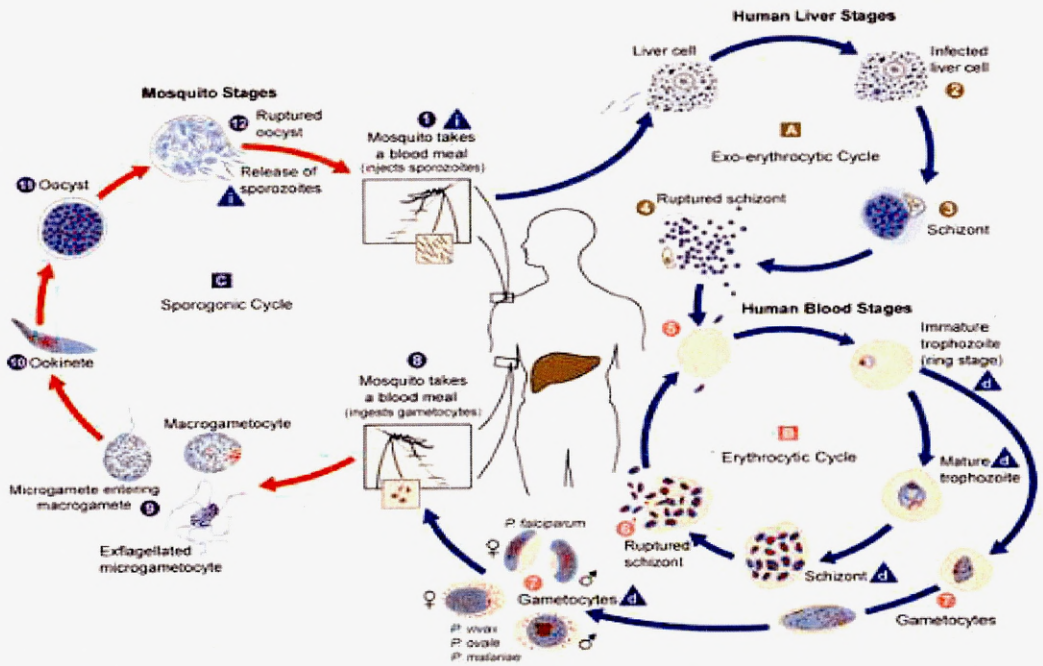

Fig. 1.2 Life cycle of malaria parasite between the vector (Anopheles mosquito) and human host. The triangular shape with an embeded 'i' represents the infective stage while the one with an embeded 'd' represents the diagnostic stage of the parasites, courtesy, CDC, 2006.

Malaria has been causing problem for mankind since time immemorial. In ancient Egyptian civilisation, it was mistaken for 'miasma' or bad air or gas from the swamp. The Chinese has been using the herb *Artemisia annua* as a prevention and treatment against malaria for the last 2000 years. In 1907, a Nobel Prize for medicine and physiology was awarded to a young French army doctor, Charles Louis Alphonse Laveran for being the first scientist to describe the life cycle of malaria Malaria is evidently as old as human civilization and man has been trying to find an effective way to combat the disease. It is not just as easy as it sound. Like many infectious diseases, malaria does not really intend to kill its host because it relies on it for survival. The parasite would run out of host to infect if it kills every host that contract the disease and would itself run out of existence (CDC,2006).

1.2.2 *Anopheles gambiae* Species Complex

An. gambiae which belong to the genus *Anophelene* is a complex of at least seven morphologically and structurally indistinguishable species discovered in the 1960s. The

complex constitutes the most important malaria vectors in sub Saharan Africa and the most efficient vectors of malaria known. The members of the specie include: *Anopheles bwambe, Anopheles gambiae sensu lato, Anopheles merus, Anopheles melas, Anopheles arabiensis Anopheles quadriannulatus*, and *Anopheles gambiae sensu stricto* (Besanky *et al.*, 1994). The members of the species despite being morphologically indistinguishable exhibit different behavioural characteristics. For instance, some members of the specie (e.g. *An. quadriannulatus*) are considered to be zoophilic (taking blood meal from animals) while others especially *An. gambiae sensu stricto* are generally anthropophilic (taking blood meal from humans). The zoophilic members are responsible for the transmission of malaria parasites such as plasmodium vivax and plasmodium ovale to animals. The methods described by Scott *et al.*, (1993) can be used for molecular identification of the members of the species but this can have important implication in subsequent control mechanisms (Fanello, Santalamazza, and Delta Torre, 2002). The interest in species identification and ecological distribution of these species is not only for academic purpose but also plays a vital role for effective malaria control measures. The identification of the species provides information on the biology of the individual species which in turn dictate appropriate control measures. For instance in 1977, there was a malaria outbreak in the south-eastern Lowveld region of Zimbabwe and members of *An. gambiae* species were collected in a benzene hexachloride (BHC) from organochlorine-sprayed dwellings. Species identification results carried out after WHO insecticide susceptibility test show that most of the survivors were members of *An. arabiensis* while most of the susceptible species were members of *An. quadriannulatus*. This result, which for the first time in Southern parts of Africa, lead to the realisation that members of *An. arabiensis* were resistant against the insecticide used, lead to a change in policy where BHC was substituted by DDT to good effect. (Green, 1981). A similar situation in South Africa in 1996 lead to DDT being replaced by pyrethroids as a

result of environmental concern mainly due to complaints by members of the community of the objectionable build up of DDT in the wall of their houses and increased bed bug biting activity. But unknown to the control program authorities, resistance to pyrethroids by the very efficient malaria vector *An. funestus* has started developing in neighbouring Mozambique. This inevitably spread across the border to South Africa and four years after replacing DDT with pyrethroids, South Africa experienced the worst malaria epidemic since house spraying was first introduces in the 1950s (Hargreaves, *et al.*, 2000; Hargreaves *et al.*, 2003). Trapping and species identification confirmed the presence of *An. funestus* inside pyrethroid-sprayed dwellings and enzyme-linked immunosorbent assays showed a *plasmodium falciparum* infectivity rate of 54% in these samples (Hargreaves *et al.*, 2000).

Studies on behavioural and distribution pattern of mosquito species in Nigeria shows that there is a variation in species and distribution pattern as one move from one part of the country to another. Although, other members of *Anopheles* species have been reported in the country, the major vectors of malaria belong to the members of *An. gambiae* and *An. funestus* complexes. Other less established species include *An. moucheti* and *An. nilli* (Gills and Coetzee, 1987). These species coexist in many parts of the country. Several studies have been conducted on *An. gambiae* complex especially in the northern part of Nigeria (Boreham *et al.*, 1979; Molineaux and Gramiccia, 1980; Hanney, 1960; Service, 1965; Rishikesh, Dideco, Petraca and Coluzzi, 1985). The ecological pattern of Nigeria shows considerable differences as one move from the south to the northern parts. The humid forest of the south turns to arid savannah, mean annual rainfall decreases, the number of dry season months increases and the vegetation become shorter and sparser (Awolola, Oyewole, Koekemoer and Coetzee, 2005). Under these climatic conditions, the mosquito breeding sites dry up more quickly, causing a rapid numerical decline in densities of mosquito. More so, cattle rearing is a major agricultural activity in this region and large cattle herds are often kept around human

habitation. Hence, the initial attraction emanating from cattle kept around human dwellings may increase the chances of humans being bitten by mosquito species that are both zoophilic and anthropophilic. These conditions may affect the abundance of host seeking mosquitoes and malaria transmission between the two parts of the country.

Awolola *et al.*, (2005) also investigated the principal vectors of malaria parasites in Nigeria and their role in malaria transmission. According to this study, species distribution in the four ecological zones of Nigeria suggests that members of *An. gambiae, An. moucheti* and *An. funestus* complexes exist in sympathy. Several other studies reported that *An. gambiae sensu stricto* is distributed all over the four ecological zones and is the most abundant. This report is consistent with previous findings that concluded that this species is omnipresent in Nigeria because of its indiscriminate breeding habits. They described it as highly endophilic, anthrapophageous, rainy season vector, but can also occasionally be zoophilic and exophilic (Okwa, Akinmolayan, Carter and Hurd, 2009).

An arabiensis has been reported in the rainforest zone, though it has been described as a major savannah vector which exists in an isolated population in deforested areas and predominantly in the dry season (Wagbatson and ogbeide, 1995). Wherever it is found in the rainforest, it is associated with a history of extensive land clearance (Hougard *et al.*, 2002). Studies by Awolola *et al.*, 2005, also reported an extensive presence of *An. arabiensis* mainly in arid zones but also in rainforest zones. A Puzzling shift in species composition of *An. arabiensis* and *An. gambiae sensu stricto* was reported in Nigeria. Another member of the *An. gambiae* specie complex, *An. melas* was found to occur mainly in the coastal rainforest and Mangroove forest. De Mellion first reported it in Lagos and was later reported by Bruce-Chwatt to be the dominant vector in the west coast being related more to sea tides (Okorie *et al.*, 2011)..

1.2.3 M and S Forms of *An. gambiae*

An. gambiae is undergoing a process of sympatric ecological speciation due to ecological adaptation to alternative larval habitat. Two morphologically indistinguishable molecular forms known as 'M' and 'S' forms, which appear to be reproductively isolated, have been described. The two forms are recognised by form-specific single nucleotide polymorphisms (SNPs) on the IGS and ITS regions of multicopy rDNA located on the X-chromosome (Caputo *et al.*, 2011). The S forms is distributed more widely across sub-Saharan Africa and breeds mostly in association with with rain-dependent pools and temporary puddles. The M form overlaaps with the S form in west and central Africa. M form showgreater ability to exploit breeding sites irrespective of seasonal variations and are closely associated with human activities such as agriculture and urbanization. This environmental adaptation allows the M forms to breed throughout the years thus sustaining year round malaria transmission (Caputo *et al.*, 2011).

observation of large numbers of polymorphic markers innitially indicated that the two forms were very highly differentiated at only a small number of discrete regions within the two autosomes and pericentromeric regions of the X-chromosome, which contain form-specific markers in the rDNA gene (Turner *et al.,* 2005). However, later observations showed that a transposable element insertion sites within the chromosome displayed marked differentiation between the forms indicating that reproduction isolation might affect larger portion of the genome (Esnault *et al.*, 2008). Genome wide studies through high density micro array SNPs analyses has recently confirmed that the molecular forms are differentiated at many loci throughout the genome in addtion to those prviously esterblished (Lawniczak *et al.*, 2010; Neafsey *et al.*, 2010; Reidenbach *et al.*, 2012). These differentiation and genetic subdivisions are very vital in vector evolution and could produce a profound influence on malaria control including insecticide resistance (White *et al.*, 2011).

differences in the adult M and S forms transcriptome appear to be minimal (Aguilar *et al.*, 2010, Casone *et al.*, 2008), but there is accumulating evidence that the larval stages are differentially adapted to particular features of breeding sites with the M forms more adapted to large areas of irrigation (Diabate *et al.*, 2008).

1.3 Insecticides and Insecticide Resistance

1.3.1 Insecticides

An insecticide is generally a pesticides used against insect pests. This definition also includes ovicides and larvicides used against the eggs and larvae of insects. Insecticides are widely used in agriculture, medicine, industries and the household. The use of insecticides has been described as one of the major factor contributing to the boost in agricultural production of the 20th century. However nearly all insecticides have potential negative side effects on the environment. Many are toxic to humans; some significantly alter the ecosystem while others are concentrated in food chain (Van Emden and Pealall, 1996).

1.3.2 Historical development of Insecticides

Ancients civilizations have used natural preparations containing inorganic compounds to control insects pests. The discovery of the insecticidal properties of dicholorodiphenyltrichloroethane (DDT) in the 1940s marked the beginning of the modern era of insecticides revolution. DDT was first synthesized by Zeidler in 1874; its insecticidal property was not discovered until 1939 (Kumar, 1984). Mass production and use of DDT began in 1943. The discovery of the insecticidal properties of DDT leads to a frenzied search for other compounds with insecticidal properties. This lead to the discovery of other classes of insecticides including carbamates, organophosphates and pyrethroids. Although, the euphoria of DDT revolution, especially in malaria control ended in the 1970s following emergence of resistance, DDT nonetheless played a vital role in malaria eradication in parts of Europe and North America.

Pyrethroids have emerged in the past several years as the major insecticides of choice in many malaria control programmes notably insecticides treated bed nest (ITNs) in many parts of the world (Wondji *et al.*, 2012) It occurs naturally as pyrethrins obtained from pyrethrum, and can also be synthesizes from oleoresin extract of dried chrysathemum flowers. The insecticidal properties of pyrethrins are derived from ketoalcoholic esters of chrysanthemic and pyrethroic acids. These acids are strongly lipophilic and rapidly penetrate many insects and paralyse their nervous system (Davies *et al.*, 2007). Both synthetic pyrethroids and pyrethrins are marketed as commercial insecticides. They are used to control insects in agriculture, in industries, homes, communities, restaurants, hospitals, schools, and many other places. They are also applied topically to treat head lice. The insecticide products are manufactured in many formulations often with addition of other chemicals known as synergists to increase potency and persistence in the environment. Pyrethrins while chemically and toxicologically similar to synthetic pyrethroids are sensitive to lights, heat and moisture. The half life is considerably reduced on exposure to sunlight making it less stable. The synthetic analogue, pyrethroids is developed to reduce these deficiencies and enhance stability. Pyrethroids like DDT works through interference with the ionic conductance of nerve membranes by prolonging the sodium current. This stimulates nerves to discharge repeatedly causing hyper excitation in poisoned animals. Both pyrethroids and pyrethrins are often formulated with oils or petroleum distillates and packed in combination with synergists, such as piperonyl butoxide (PBO) and n-octylbicycloheptane dicarboximide (Davies *et al.*, 2007).

1.3.3 Classification of Insecticides

The table below summarizes the major classes od insecticides, thier active ingredients and their mode of actions.

Table 1.1 Major insecticides classes, thier active ingredients and mode of actions

Insecticide Class	Activie Ingredients	Mode(s) of Action
Carbamates	Aldicarb, Bendiocarb, Methiocarb, Carbaryl, Oxamyl, Propoxur, Carbofuran, Methomyl, Thiocarb, etc.	Inhibitors of Acetylcholine esterase
Organophosphates	Chlorpyrifos, Diazinon, Ethion, Ethoprop, Dimethoate, Acephate, Fenthion, Naled, Isofenphos, Temephos, Oxydemeton-methyl, Trichlorfon, Parathion, Sufotep, Chlorethoxyfos, Chlorpyrefos, etc.	Inhibitors of Acetylcholine esterase
Cyclodiene organochlorines	Endosulfan, Chlordane, and Gamma-HCH (Lindane)	Antagonist of GABA-gated chloride channels
DDT	DDT	Modulator of sodium Ion-gated channels
Pyrethroids/Pyrethrin	Allethrin, Bifenthrin, Deltamethrin, Permethrin, Phenothrin, Tetramethrin, Cyfluthrin, Cycloprothrin, Cyhalothrin, Cypermethrin, Acrinathrin, etc.	Modulator of sodium Ion-gated channels

1.3.4 Insecticide Resistance and its Development

Insecticide resistance is defined as a marked reduction in the sensitivity of an insect population to insecticides. The first case of insecticides resistance was documented in 1914 by A. L. Melander when inorganic insecticides resistance was discovered in scale insects. Eleven additional inorganic insecticides were reported between 1914 and 1946 to be losing their sensitivity and effectiveness against insect control. The development of organic insecticides such as DDT in the early 1940s lead to a shift of attention from inorganic to organic insecticides, a decision which was thought would solve the problem of insecticide resistance. This hope was never realised. In 1947, barely a year after it was used, the first case of resistance to DDT was documented in housefly. Since then, many more synthetic insecticides have been developed almost all of which have been rendered ineffective by resistance evolution.

Insecticides resistance constitutes a serious threat to public health because insecticides play a central role in the control of major disease borne vectors such as mosquito (Balkew *et al.*, 2012). The WHO launched malaria eradication program in 1955 with house spraying of DDT. In the beginning, it was successful with a marked decrease in vector density and a consequent fall in malaria mortality and morbidity. But this euphoria soon ended when mosquitoes develop resistance to DDT forcing WHO to abandon the program in 1976 and replaced it with malaria control. This malaria eradication program together with advances in environmental management and rapid infrastructural development was actually responsible for malaria eradication in many parts of Europe and North America. But the little gain made in Europe and America was to constitute an enormous consequence for malaria control in other parts of the world especially in Tropical Africa where the highest burden of malaria lies (Hemingway and Ranson, 2000). The speed with which mosquito develop resistance to in particular, DDT was amazing. The compound was first introduced in 1946 and a year later in

1947, the first case of resistance was discovered in *Aedes tritaeniorlynchus* and *Aedes solicitarus*. Since then many other mosquito species have followed suit and as at present, more than 100 species of mosquito have developed resistance to almost all the classes of insecticides used for their control and more than 50 of these species are in the *Anopheline genera* (Wondji *et al.*, 2012). Over the years after development of resistance to DDT, many classes of insecticides have been employed in malaria control. These include organophosphates, BHC, carbamates and most recently pyrethroids which are used as indoor residual spray (IRS) as well as insecticide treated bed nets (ITNs). Resistance have developed to most of these classes of insecticides (Hemingway and Ranson, 2000). Particularly worrisome is recent evidences of resistance to pyrethroids in African malaria vector, *An. gambiae* given the recent emphasis by WHO and other international health agencies on the use of pyrethroids for impregnated bed nets in their roll back malaria campaign (Lengeler, 2004).

1.3.5 Mechanisms of insecticide resistance

The mechanisms of insecticides resistance in insect vectors are molecular and biochemical in nature. Generally, two major mechanisms have been identified which include target site mechanism and detoxification mechanism. In target site mechanism, the insecticides can no longer bind to its target site while detoxification mechanism involves increase in the levels or activities of metabolising enzymes. This prevents the insecticides from reaching its site of action. An additional mechanism has been proposed, base on thermal stress response, but its importance and contribution to the overall resistance spectrum has not yet been assessed (Patil, Lole and Deobagker, 1996).

1.3.5.1 Target Site Resistance Mechanism

Mutations which lead to changes or alterations of amino acids responsible for insecticide binding at its site of action cause the insecticide to become less active or even total loss of activity. Acetylcholinestrase in the nerve synapses is the binding site for organophosphorus such as malathion, fenitrothion, and carbamates (e.g. propoxur and sevin) while DDT and synthetic pyrethroids binds to the sodium ion gated channel in the nerve shealth. DDT-pyrethroid cross resistance has been observed, involving a single amino acid changes (fig.1.3) in the axonal sodium channel insecticide binding site (Ndiath *et al.*, 2012). This type of cross resistance appears to produce a shift in sodium current activation curve and cause low sensitivity to pyrethroids (Davies *et al.*, 2007). Single nucleotide change within the gene coding for γ-gamma glutamyl receptor also appear to be responsible for resistance to dieldrin (Davies *et al.,* 2007; Santolamazza *et al.*, 2008). Researchers have already identified at least five point mutations in the acetylcholinesterase binding site that may confer varying degree of resistance to organophosphorus and carbamates classes of insecticides (Hemingway *et al.*, 2004; Edi *et al.*, 2012).

1.3.5.2 Detoxification Mechanism

The enzymes esterases, glutathione-S- transeferases, and P450 oxidases are responsible for insecticide detoxification or metabolism in living organisms. These enzymes are transcribed by large multigene families. Elevated levels or activities of these enzymes appear to be the most common resistance mechanism in insects (Hemingway *et al.*, 2004). The P450s are heme-containing family of enzymes distributed widely in insects. They are involved in the metabolism of wide range of environmental xenobiotics including many classes of insecticides. The role of P450s in the metabolism of pyrethroid insecticides has been particularly studied extensively in *An. gambiae*. Vulule *et al.*, (1999) first demonstrated the

involvement of P450s in pyrethroid resistance in *An. gambiae* in Kenyan villages by displaying increased heme levels of P450s in resistant mosquitoes. This was followed by demonstration in 2003 that CYP6Z1 was overexpressed in a pyrethroid-resistant *An. gambiae*, giving an indication of the family of P450 involved in pyrethroid resistance (Nikou *et al.,* 2003). Later study (David *et al.*, 2005). involving microarray detoxification chips resulted in the identification, among other genes, more P450s that were highly expressed in permethrin and DDT-resistant strains of *An. gambiae* including CYP325A3, which belong to a class that was not initially associated with insecticide resistance. Awolola *et al.,* (2009) also used the same approach to report a constitutive over-expression of CYP325A3 in a permethrin-resistant Nigerian *An. gambiae*. In addition, CYP6M2, CYP6Z2, and CYP6Z3 were identified as being associated with pyrethroid resistance in Ghanaian populations of *An. gambiae* (Muller *et al.*, 2007). A follow up study however showed CYP6Z2 as having broad spectrum substrates specificity and could be involved in the metabolism of many other xenobiotics other than insecticides (McLaughlin *et al.*, 2008). Other studies (Djouaka *et al.,* 2008; Muller *et al.,* 2008) identified another isoform; CYP6P3 and further confirmed CYP6M2 as being up-regulated in many permethrin-resistant strains of *An. gambiae.* The expressed recombinant proteins of these genes confirmed their capacity to metabolise permethrin and these genes are usually over-expressed in permethrin-resistant strain of *An. gambiae* (Stevenson *et al.,* 2011). Pyrethroid is not the only insecticide implicated with the activities of P450s, for some cytochrome P450 families have been reported to be over-expressed in a DDT-resistant *An. gambiae*. Over-expression of CYP6Z1 and CYP12F1, together with other detoxification genes, was reported in a DDT-resistant *An. gambiae*, suggesting that P450s could also play important role in the detoxification of DDT (David *et al.*, 2005). Finally, P450s were established as a major contributors to insecticides resistance by many species of insects by RNAi silencing of the main electron donor and coupling

partner of P450s, the POR. *An. gambiae* with knock down POR were found to be highly susceptible to permethrin (Lycett *et al.*, 2006).

Glutathione S-Transferase (GST) is another major class of detoxification enzyme system which play important role in the metabolism of many insectides. Elevated levels of this enzyme has been implicated in many incidences of resistance. This increase in GST activity was believed to be due to increased levels of one or more GST isoenzymes arising either through gene amplification or the most commonly increase in gene transcription. Qualitative changes in individual enzymes was earlier thought to be a contributing factor, however. recent insights suggested this not the case (Ranson and Hemingway, 2004). The first realizations that increase in the activity of GST could be attributed to the incidence of insecticides resistance in many insect species was made in studies involving organophosphate insecticides. It is now established that resistance to organophosphate insecticides in many insect species is as a result of changes in the activities of GST (Enayati *et al.*, 2005). Studies on tolerance to another major class of insecticide, DDT, by various species of insects was never initially associated with activities of GST. This was because the major enzyme responsible for dechlorination of DDT to produce dichlorodiphenylethylene (DDE) was never thought to be part of the GST family. Not until several chlorodinitrobenzene (CDNB) conjugates of this enzyme were studied in DDT resistant populations of housefly did it reveal their peculiar characteristics as a member of the GST family (Clark and Shamaan, 1984). Elevation of the activities of GST has been confirmed as one the major mechanisms of resistance to DDT by insects (Hemingway *et al.*, 2004; Edi *et al.*, 2012). A direct role has not been established for GST in the metabolism of pyrethroid but GST could nonetheless play a very important role in conferring insects resistance to pyrethroid by serving as a detoxification route for products of lipid peroxidation produced during the metabolism of pyrethroids (Vontas *et al.*, 2001).

Several studies have implicated the activities of CEs in insecticides resistance in many insect species (Hemingwat *et al.*, 2004; Cui *et al.,* 2011). The mechanisms reported in many of these studies involved increased activities of the enzyme leading to increased accumulation of hydrolytic products in resistant compared to susceptible strains of insects (Hemingway *et al.*, 2004). Elevated levels of esterases can be detected using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or immunoassays in resistant insect strains. Alternatively, biochemical enzyme assays have been developed employing α and β- naphthyl acetate as substrates to investigate esterase activities in a variety of insect strains (WHO, 1998).

1.3.5.3 Resistance to Growth Factors, Ivermectins and other Microbial Agents.

Growth factors, ivermectins and toxins from microorganisms were recently introduced into vector control. In many cases, the mechanisms of action as well as resistance development are not yet well understood. However, the mechanisms that confer resistance to growth factors were thought to be oxidase-based (Kotze *et al.*, 1997). A number of mechanisms such as oxidation, conjugation and target site alteration were thought to be responsible for conferring resistance to ivernectins (Clark *et al.*, 1995). Many insect vectors have not yet developed resistance to these compounds in the field. Biological approach to vector control involved toxins from bacteria showing insecticidal activities. Examples are *Bacillus thurigiencies israelensis* and *Bacillus sphaericus*. The mechanism of *Bacillus sphaericus* toxin as a biological insecticide is not yet understood (Rao *et al.*, 1995; Rodcharoen and Muller, 1996), although it was believed that several interconnected mechanisms are involved (Nielsen-Leroux *et al.*, 1997). On the other hand, resistance to *Bacillus thurigiensis israelensis* toxin is thought to result from the reduced binding of the toxin to the brush border in the lumen of the insect gut (Escricle *et al.*,1995; Tabashnick *et al.*, 1996) or by increased digestion and metabolism of the toxin by gut proteases (Keller *et al.*, 1996). Six different

types of toxins from several strains of *Bacillus thurigiensis israelensis* are being used in vector control to prevent or forestall the development of resistance; however multitoxin resistance has already been reported (Tabashnik *et al.*, 1997; Cheng *et al.*, 1997).

1.3.6 Genetic and Molecular Basis of Insecticides resistance

Malaria vector control make use of several types of insecticides all of which belong to four chemical classes targeting two neurological sites in insects. Synthetic pyrethroids and DDT share the same site; the voltage-gated sodium ion channel while organophosphates and carbamates acts on the acetylcholinesterase receptor. The result of similarity in target sites among various classes of insecticides is the development of knock down resistance (kdr) mutations by various insect species including *An. gambiae*. Several authors in East and West Africa have presented evidences of a strong correlation between the expression of the resistance phenotype (as measured by insecticide exposure assay) and genotype at the kdr locus in *An. gambiae* mutations (Verhaeghen *et al.*, 2006); Corbel *et al.*, 2007; Santolamazza *et al.,* 2008). A transition mutation involving the substitution of leucine with phenylalanine was observed in the west African mutation while the east African mutation involve the substitution of leucine with serine (Fig.1.3). These compelling associations have lead to the development of molecular diagnostic tools for the detection of kdr mutations (Lynd *et al.,* 2005; Syafruddin *et al.*, 2010; Singh *et al.*, 2011).

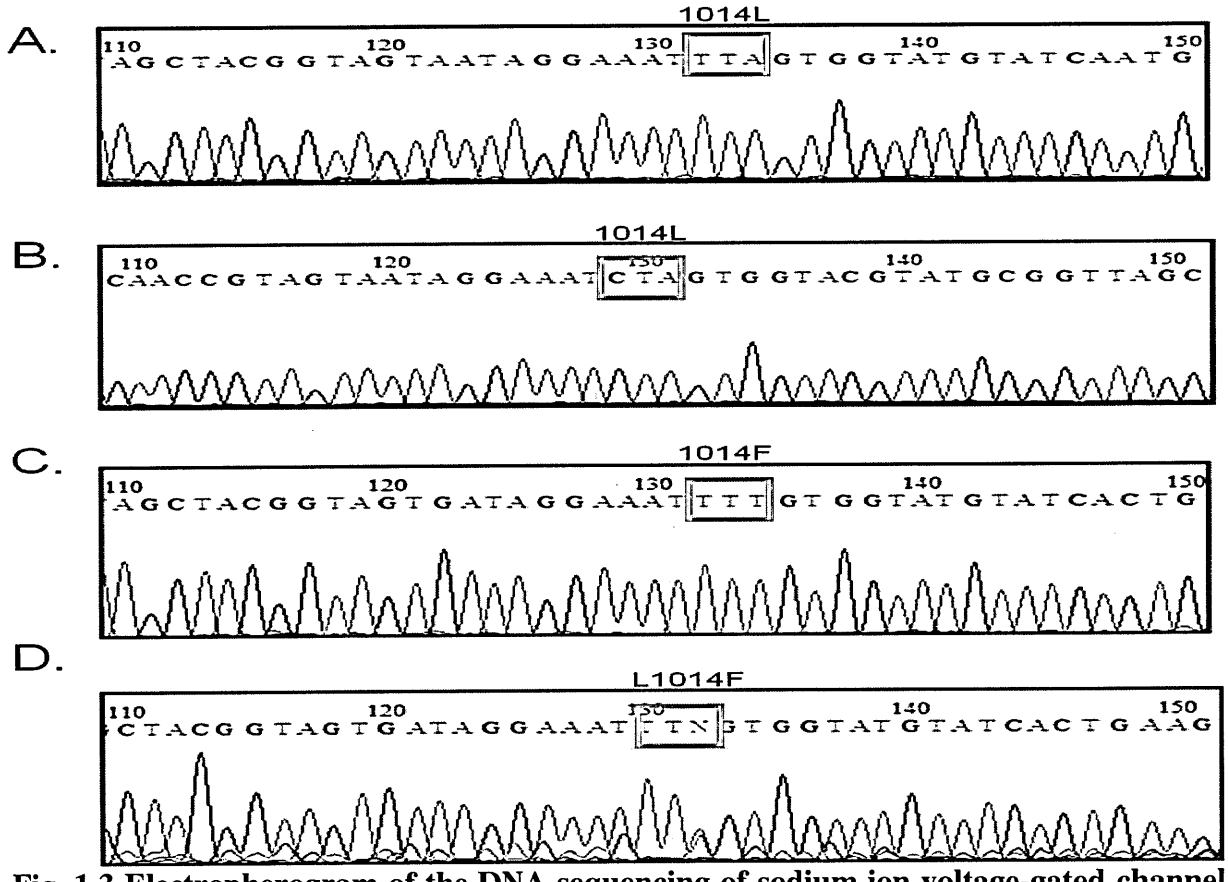

Fig. 1.3 Electropherogram of the DNA sequencing of sodium ion voltage-gated channel gene fragment from *An. tesselatus* (A), *An. balabacensis* (B), *An. sundaicus* (C and D) from Indonesia. A and B indicate the wildtype allele, 1014L (TTA and CTA). C indicates resistance allele1014F (TTT), and D indicates the mixed allelic types between L/F. Adapted from Syafruddin *et al.*, 2010.

Over the past several years, molecular basis of metabolic resistance, involving the activities of the major detoxification enzymes has also been elucidated. Most, but not all of these metabolic based resistance mechanisms involve vectors displaying increased levels or activities of detoxifying enzymes compared to their susceptible counterpart. Gene amplication and trascriptional up regulation underlies the major molecular mechanism of insecticide resistance resulting in both qualitative and quantitative changes in many detoxification enzymes (Hemingway *et al.*, 2004). With the advent of next generation sequencing technology, several trans-acting regulatory elements upstream of many of the cloned detoxification enzymes and other candidate regulatory genes mediating the activities

of several detoxification genes have been identified (Cui *et al.*, 2011; Chung *et al.*, 2012; Saavedra-Rodriguez *et al.*, 2012; Jaramillo-Gutierrez *et al.*, 2010).

1.4. Environmental Xenobiotics and Insecticides resistance

1.4.1 Xenobiotic Pollution and Insecticides Resistance in Insects

Resistance or insecticides tolerance has evolved in many insect species due to long period of insecticide application for disease control. Studies have however shown that other processes such as environmental pollution and misuse of pesticides also contribute to the evolution and perpetuation of insecticides tolerance. Environmental pollution resulting from industrial activities, agriculture, mining etc has lead to changes in organisms. These changes occur at various levels of organisms such as cellular level, tissue level, at the level of the organism, at population level as well as at the level of the ecosystem. As a result of pollution, insects are under pressure to respond to changes in the environment in order to survive. These changes can take many forms; number of insects may change as result of changes in their vital capacity and fecundity (Golutvin *et al.*, 1981; Heliovaara and Verzanen, 1994), also Populations of insects resistant to insecticides may appear as a result of selection pressure posed by pollution (Lauridsen and Jersperson, 1997). One of the first organisms to develop resistance as a result of environmental pollution and insecticide pressure is the house fly, *Musca domestica* (Keiding and Jespersen, 1991). Observations from other insect species have demonstrated the contribution of environmental xenobiotics to the development of resistance to insecticides used for their control. For instance, Boyer *et al.*, 2006 reported that *Aedes aegypti* larvae exposed to the herbicide atrazine become more tolerant to the organophosphate temephos. Similarly, exposure of *Aedes albopictus* larvae to benzothiazole and pentachlorophenol can increase their tolerance to insecticides such as carbaryl, rotenone and temephos (Suwanchaichinda and Brattsen, 2001; Suwanchaichinda and Brattsen, 2002).

Induction of detoxification enzymes such as P450, GST and non specific esterases in response to various environmental chemicals or xenobiotics have been reported in many insect vectors. Also, studies have established correlation between increase in tolerance to various classes of insecticides in many insects and induction of detoxification enzymes as a result of prior exposure of insects to environmental chemicals (Feyereisen, 2005; Hemingway *et al.*, 2002; Hemingway, *et al.*, 2004). Drosophila and *Aedes aegypti* have featured more prominently than other insects species in model studies involving induction of detoxification enzymes as a result of prior exposure to environmental xenobiotics, and incidences of toletrance to various classes of insecticides (Le Goff *et al.*, 2006; Poupardin *et al.*, 2008). Finally, mechanisms underlying the induction of detoxification enzymes in response to environmental xenobiotics including insecticides have been well documented (Luo *et al.*, 2004; Vontas *et al.*, 2005; Backlund and Ingelman-Sundberg, 2005; Petrulis and Pardew, 2002; Brown *et al.*, 2005; Gilbert, *et al.*, 2002; King-Jones *et al.*, 2006 and Li *et al.*, 2002).

1.4.2 Agro-allied Chemicals and Insecticides Resistance

Most of the studies on insecticides tolerance in insects have focused primarily on insecticides used for public health programmes. This is understandable since the major objective is to control disease carrying vectors in order to guarantee public health safety. However, the fact that some of these insecticides or those chemically similar to them are also applied in agriculture, and the gradual emergence of agriculture as an increasingly resource intensive enterprise has necessitated a consideration of the role of agricultural practices in the evolution and development of insecticides resistance by public health vectors (Overgard, 2006). A case for agricultural involvement in insecticides resistance was first made when resistance to DDT and the now obsolete dieldrin, appeared in *Anopheline* mosquitoes in Greece and Africa as a result of agricultural treatment of cotton and rice (Hamon and Garrett-Jones, 1963). Later studies also implicated agricultural practices in the development of multiple resistance

involving DDT, dieldrin (DI), organophosphates, and carbamates also in *Anopheline* population in countries such as Turkey, India and Central America. The role of agriculture in the development of insecticides resistance by public health vectors is not new, but few attempts have been made to evaluate the extent of involvement of various agricultural practices in development of resistance. The evidence is more noticeable in places like Southeast Asia where agriculture is becoming more resource intensive and vector-borne diseases are rampant, and where both often coincide spatially (Overgaard, 2006).

Over 90% of insecticides produced globally are used in agriculture as pesticides. Agricultural usage of insecticides can exert selection pressure on disease vectors at different stages of their development especially the larval and adult stages. Agricultural practices such as rice fields and irrigation schemes create breeding sites for these vectors which are sprayed with insecticides during treatment. Wind blowing in the direction of breeding sites can transfer sprayed insecticides to mosquitoes and rainfall can wash away insecticides applied on farmlands into pools and water bodies where mosquitoes are breeding. Some species of mosquitoes such as *An. pharoensis* have been found to be resting on insecticides treated trees. Contamination of mosquito breeding sites by agricultural insecticides subjects mosquito larvae to selective pressure which is more likely to induce resistance more rapidly than house spraying of adult mosquitoes which reached only anthropophilic females which are generally less than 25% of the total mosquito population for semi exophilic species. Larval exposure can trigger genetic selection for resistance by the action of insecticides residues acting at sub lethal doses (Tia *et al.*, 2006).

Relationship between agricultural insecticides and insecticides resistance in mosquitoes has been used to categorise agricultural insecticides selection pressure on mosquitoes into; (1) Those mosquitoes which develop resistance before chemical vector control (2) mosquitoes found in agricultural areas displaying higher resistance than those found in non agricultural

areas (3) vector resistance developed during agricultural spraying (4) measure of the degree of correlation between the intensity of insecticides usage in agriculture and degree of resistance in vectors (5) correspondence between the spectrum of cross resistance in vectors and the types of insecticides applied to crops and (6) temporary suppression of vector population after agricultural use of insecticides. Some of these points may be purely circumstantial, and since in many cases, both agricultural and public health insecticides are used in the same area, it is sometimes difficult to reach a definite conclusion with absolute certainty (Overgard, 2006). However several studies (Tia *et al.*, 2006; Overgard *et al.*, 2002; Overgard, 2006; Diabate *et al.,* 2002) using both fiel populations and laboratory models have demonstrated resistance to several classes of insecticides as a result of agricultural selection pressure in many parts of the word. The future of malaria control especially in the developing countries of sub Saharan Africa lies with the use of pyrethroids both as residual in-house sprays and in insecticides treated bed nets. This means that there is an urgent threat to this approach since pyrethroids is also one of the main pesticides of agricultural control of pests. In fact the amount of pyrethroids used in public health vector control is very small compared to the amount used in agriculture. Already in many places, evidences of pyrethrods resistance have been reported, but the contribution of agriculture to this development has not been largely evaluated. Several African countries have all reported pyrethroid resistance (Awolola *et al.*, 2005; Awolola *et al.*, 2009; Ahoua Alo *et al.*, 2012; Protopopoff *et al.*, 2013) in many mosquito vectors. There is no clear evidence of how this resistance developed, agricultural use of pyrethroids might be responsible, in particular in places where small scale irrigation practices are being undertaken. This kind of selection pressure is almost impossible to control. Cross resistance may also be a problem. In areas where organophosphates are predominantly used for vector control, organophosphate-pyrethroid cross resistance may develop and compromise the use of pyrethroids for vector control. Thus the use of

insecticides in agriculture must be considered a real threat even to pyrethroid use of vector control initiative (Protopopoff, 2013).

The dynamics of resistance development in vectors is complex and varies from species to species. But the fundamental requirement for resistance development is exposure to insecticides and genetic variation in insecticides susceptibility. Assessing the geographical extent of insecticide exposure is relatively easy compared to assessing the geographical extent of genetic variability. It is therefore necessary to develop maps of overlapping intensive insecticides cropping areas and areas of high endemicity of diseases. Such maps can be used to identify risk areas for resistance development in response to agricultural activities. Identifying risk areas on the other hand will be helpful in assessing potential locations for the implementation of resistance management and integrated control strategies such as reducing and replacing insecticides used in agriculture or for public health control measures as well as coordinating integrated pest and vector management (Overgaard, 2006). The need for integrated pest and vector management and control was expressed at UNEP/FAO/WHO workshop on 'Sustainable Approaches for pest and vector management and Opportunities for Collaboration in Replacing POPs Insecticides' in 2000. It was agreed that an inventory identifying target areas and pilot field for studies and training of personnel on integrated pest and vector control will contribute abundantly to the implementation of action plans on resistance management especially through coordinated approaches between different sectors involved in resistance management and control. The management of agroecosystems varies from one country to another and from region to region. It also depends on the insecticides in focus. Mapping areas of possible vector resistance as a result of agricultural treatment of insecticides can be done in several ways all of which depends on the availability and quality of background data and its interpretation.

1.4.3 Hydrocarbon/Petrochemicals and Insecticides Resistance

Petroleum or fossil fuel is a natural resources formed as a result of decomposition of organic matter at high temperature over a period of millions of years and is a complex mixture of gaseous, liquid and solid hydrocarbons. Products from processing of crude oil constitute about 50% of world's energy, transportation, electrical utility and heating requirements. Petroleum products also acts as substrate for the manufacture of a wide range of industrial products like lubricants, insecticides, solvents, the surfacing of highways, waterproofing, and in the manufacture of plastics and feedstock. The impact of oil and gas exploration and processing on the environment takes various forms and dimension. Apart from isolated and accidental incidences such as oil spills which contaminate land and water bodies, petrochemicals also come into contact with the environment through pipeline leakages, urban input, industrial emissions, as well as domestic and commercial uses (Ou *et al.*, 2004). The major compounds from petrochemical industries release into the environment can be classified into many categories including polycyclic aromatic hydrocarbon (PAHs), aliphatic hydrocarbon and biomarkers (Wang and Stout, 2007). The impact of pollution from these compounds affects plants, animals as well as man. Apart from the 'supposed' contribution of oil and gas pollution to oxone layer depletion and global warming, various research effort have been dedicated to the effect of oil and gas pollution on specific biotic and abiotic components of the ecosystem with the attendant impact on human health. In Nigeria for instance, Ekom (2006) and Adekunle *et al.*, (2010) investigated the toxic effect of used engine oil on phytoplankton species of the Calabar River Estuary while Obire and Anyawu (2009) investigate the effects of various concentrations of crude oil on fungi population of soil. Microalgal bioindicator for crude oil pollution, methods of bioremediation and the levels of petroleum hydrocarbon in some organisms found in Qua Iboa Estuary were investigated in Nigeria (Essien *et al.*, 2006; Essien, *et al.*, 2007; Benson *et al.*, 2008). Another study

(Uwuanyanwu *et al.*, 2011) using laboratory animal models also established the toxic effect of some refined petroleum products such as petrol and kerosene contaminated diet on some body organs such as the liver and the kidney by measuring the levels of enzymatic markers of liver and kidney toxicity. The result of these study shows considerable damage to liver and kidney cells following exposure (Patrick-Uwuanyanwu *et al.*, 2011).

Most of the data available on the impact of petroleum hydrocarbon on the ecosystem are focussed primarily on carbon emission from petrochemical industries and its contribution to global warming and green house effect as well as the toxicological studies of the effect of petrochemicals on many organisms especially marine organisms exposed to oil spills in various parts of the world. Only few researches have been carried out so far to evaluate changes in behaviour of terrestrial organism exposed to petrochemical contamination. Furthermore, so far to my knowledge, no studies have been conducted to investigate the impact of petrochemicals in the environment as potential selection factors for the emergence and development of insecticides resistance in insect species despite the fact that some of the synthetic insecticides are produced from the by-products of many petrochemicals. This situation is surprising especially in places like Nigeria and other Sub Saharan African Countries where it is a normal traditional practice to treat stagnant water bodies with refined hydrocarbon products such as kerosene, petrol and spent oil to control mosquito larvae. Consequently, this traditional practice was part of what informed the choice of some aspect of this research (Pascom, 2010).

However, studies (Ana *et al.*, 2009; Van de Oost *et al.*, 2003; Lima *et al.*, 2007; Guimareas *et al.*, 2009; Martinez-Gomez *et al.*, 2006 and Martinez-Gomez *et al.*, 2009; Lee and Anderson, 2005) have established induction of the three major detoxification enzymes; Carboxyl esterases, Glutathione-S transferases and Cytochrome P450 oxidases to levels of petroleum hydrocarbon in some organisms found in contaminated environments. Data from these kinds

of studies could be use to draw inferences and formulate hypotheses for the impact of environmental petrochemicals on metabolic changes in insect since most of these detoxification genes are highly conserved across many organisms.

Much emphasis has been placed on the effect of polycyclic aromatic compounds in many studies involving impact of petrochemical pollution on the ecosystem. This is because polycyclic aromatic hydrocarbons (PAHs) are a major environmental pollutant resulting from both petrogenic and pyregenic sources. Its occurrence in the environment is largely due to burning of fossil fuel, exhorts from motor vehicle, burning of waste and oil refining (Mc Elroy *et al.*, 1989; Srogi, 2007). Another study investigated the effect of five members of PAHs containing more than one aromatic ring on the activities of Glutathione-S-Transferase and Ethoxyresorufin-O-Deethylase in Nile Tillapia (*Oreochromis niloticus*) which is considered a potential fish species for biomonitoring pollution in aquatic environments. Like in many other related studies, the result showed that some members of PAHS especially those with two to four aromatic rings are strong inducer of GST, though in this study, the induction does not appear to be dose dependent (Pathiratne and Hemachandre, 2010). Similarly, induction of a P450 gene (CYP1A) in response to levels of PAHs was reported in red mullet (Della Torre *et al.*, 2010) and harlequin ducks (Esler, *et al.*, 2010) and these inductions were found to correlate with levels of PAHs in the environment.

1.5 Aim and Objectives

The aim of this PhD study is to investigate the role of environmental physico-chemical factors, present in mosquito breeding sites, as sources of selection pressure for the emergence and development of insecticide resistant mosquitoes in Northern Nigeria. The hypothesis is that since mosquito breeding sites are found in varieties of ecologies where different human related activities are taking place, environmental chemical compounds arising from such

activities are taken into any surrounding mosquito breeding sites. Therefore, prior exposure of *An. gambiae* mosquito thriving in such environments to these arrays of chemicals could act to select them for resistance to public health insecticides after exposure, especially those compounds having similar structures and activity relationships with different classes of insecticides used in malaria control. The specific objectives include:

1). To prospect for, and conduct sampling of *An. gambiae* larvae from breeding sites located in different ecologies categorized and grouped into three different study zones in towns of villages spread out across Kano and Jigawa states of Northern Nigeria. These study zones include; Zone A (intensive agricultural areas), Zone B (domestic and residential environments) and zone C (areas of commercial activities where petrochemical/hydrocarbon products are sold, processed, used, and/or discharged).

2). To determine the *An. gambiae* and any other mosquito species larval density per litre of breeding water.

3). To measure the levels of the physical environmental factors; pH, temperature, conductivity, transparency total dissolved solids, dissolved oxygen, biological oxygen demand from the mosquito breeding sites and the chemical environmental factors; sulphates, phosphates, nitrites, nitrates, carbon content and oil and grease from water samples taken from the mosquito breeding sites.

4). To carry out quantitative assays of detoxification enzymes; P450 monooxygenase, Glutathione-transferase and non specific esterases on the three life stages of *An. gambiae* mosquito sampled from all the breeding sites visited across the three study zones.

5). To determine the levels of the various forms of glutathione (total, oxidized and reduced) from the three life stages of *An. gambiae* sampled from all the breeding sites across the three study zones.

6). To use appropriate statistical tools and packages to investigate relationships and correlations between the physico-chemical factors and *An. gambiae* larval density, between the physico-chemical factors and the detoxifications enzymes activities and between the various forms of glutathione and the activities of the detoxification enzymes across the three life stages of *An. gambiae*.

7). To use observations from 1-6 above to describe the impact and importance of this study to the contemporary malaria management and control initiatives and to make suggestions, based on these observations, on novels strategies and approaches that could tackle the challenges facing the current malaria management programmes.

CHAPTER TWO

2.0 Materials and Methods

2.1 Chemicals and Reagents

All the chemicals and reagents used in this study were of analytical grade and were purchased from Sigma-Aldrich (Dorset, United Kingdom) and BDH chemicals (VWR International Ltd. Leicestershire, England, United Kingdom) unless otherwise indicated.

2.2 Assessment of physico-chemical environmental factors

Levels of seven physical environmental factors in the mosquito breeding water were determined. These factors were pH, temperature, conductivity, transparency, dissolved oxygen (DO), biological oxygen demand (BOD) and total dissolved solids. pH, conductivity, temperature, transparency and total dissolved solids were measured on-site during larval collection, while DO and BOD levels in the water samples collected from the mosquito breeding sites were determined in the laboratory. Furthermore, the concentrations of six chemical environmental factors were also determined from the water samples taken from all the *Anopheles gambiae* breeding sites visited across the three studied zones. The chemical factors evaluated were sulphate, phosphate, nitrite and nitrate ions as well as carbon content, and oil and grease. The methods followed and/or established for all these determinations are presented below.

2.2.1 pH, Temperature, Conductivity, and Total Dissolved Solids

Measurement of pH, temperature and conductivity was carried using the COMBO PH/EC/TDS/Temperature metre (HI98129 from HANNA Instruments, United States). The instrument's operational instruction (See Appendix) was followed. The metre was initially calibrated according to the manufacturer's instructions. For pH measurement, the pH mode

was selected by holding and pressing the SET/HOLD button on the Combo metre. The metre probe was then submerged into the mosquito breeding water and measurements taken from the figure displayed on the primary LCD after the stability symbol on the top left of the LCD disappeared. The pH value was automatically compensated for temperature, whose measurement was also taken from the figure displayed on the secondary LCD of the COMBO pH/EC/TDS/Temperature metre. Measurements were taken from different points in the breeding water.

The same procedure as in pH was followed to measure electrical conductivity (EC). After pH measurement, the metre was recalibrated with the calibration solution for EC (HI7031) after changing the metre to EC mode according to the manufacturer's instructions (See Appendix). To measure the EC, the metre probe was submerged into the mosquito breeding water and measurements taken on the primary LCD after the stability symbol on the top left of the LCD disappeared. The temperature of the breeding water is also displayed on the secondary LCD and was also recorded again. Temperature and EC measurements were expressed in degree Celsius (°C) and as microsemile per centimetre (μS/cm) respectively.

The Combo PH/EC/TDS/Temperature metre (HI 98129 model from HANNA Instruments, United States) was also used to measure the levels of total dissolved solids in the *An. gambiae* breeding water in a similar procedure as followed for pH, EC, and temperature measurements. The concentration of TDS in the mosquito breeding water was expressed in mg/L.

2.2.2 Transparency (Maiti 2004)

A 25 cm in diameter white/black Secchi disc attached to a graduated rope was used to measure the transparency of the mosquito breeding water as described by Maiti (2004). The disc was lowered into the breeding water until it disappeared from view. The point on the

rope at the upper surface of the water was noted and designated L_1. The disc was then lowered a little further and then lifted slowly until it reappeared. The point on the rope at the upper surface of the breeding water was also noted and labelled L_2. Transparency was calculated as follows: L1 + L_2/2. Three measurements were taken at different points in the breeding water and the average calculated to the nearest centimetre.

2.2.3 Dissolved Oxygen (DO) and Biological oxygen demand (BOD)

The procedure followed for DO and BOD determination was as described by Maiti (2004) with slight modification. The DO in the mosquito breeding water samples was taken with DO probe metre (Hach Lange, HQ40d model, Loveland, Colorado-United States) and the readings recorded as mg/L. The BOD test was prepared by using distilled water containing 1 ml of phosphate buffer, magnesium chloride, calcium chloride and ferric chloride per litre of water (this provide the sample with pH buffering, osmotic balance and essential nutrients) as diluent to dilute the water samples taken from the mosquito breeding sites. The diluted mosquito breeding water sample (1 in 1 dilution) was then transferred into the BOD bottle and closed immediately with a stopper. Initial DO measurements were taken using the DO metre and recorded as DO_1. The samples were then incubated at 20°C for five days and the final dissolved oxygen (DO) readings were taken and recorded as DO_2. The BOD of the mosquito breeding water samples was calculated as follows:

$$BOD_5\ (mg/L) = \frac{DO_1 - DO_2}{P}$$

Where P = decimal fraction of the dilution water used.

$$P = \frac{\text{Volume of mosquito breeding water}}{\text{Volume of mosquito breeding water + dilution water}}$$

2.2.4 Determination of sulphate ion concentration

Sulphate ions concentration in the mosquito breeding water samples was determined by Turbidimetric method using APHA, 1995 standard procedure as described by Maiti (2004). Firstly, conditioning reagent was prepared by mixing 50 ml glycerol with a solution containing 30 ml concentrated HCl, 300 ml distilled water, 100 ml isopropyl alcohol, and 75g NaCl. Then, 100 ml standard sulphate solutions with five concentrations; 5 mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml and 25 mg/ml were prepared from a stock sulphate solution of 100 mg/ml. After the preparation of the standard sulphate solutions, 20 ml of each sulphate standard solutions and 20 ml of filtered mosquito breeding water samples were added into Erlenmeyer flask and the volume made up to 100ml with distilled water. Then 5 ml of the conditioning reagent was added to both the samples and to each of the five standards and mixed gently with the magnetic stirrers. While the solutions were being stirred, 0.3g of $BaCl_2$ was weighed and added to each of the mixtures. The solutions were stirred further for 1 min and then allowed to stand for 5 min. The absorbance at 420nm of all standards and test samples against distilled water blank were recorded using specttrophotometer (Jenway 6305 model, Bibby Scientific Ltd. Staffordshire, UK). An absorbance vs Concentration standard curve was created for the five standard solutions. Finally, the concentration of the SO_4^{2-} present in the test samples were estimated off the standard calibration curve.

Calculation: $\text{mg/L } SO_4^{2-} = \dfrac{\text{mg } SO_4^{2-} \times 1000}{\text{mL of sample}}$

2.2.5 Determination of phosphate ion concentration

The Stannous-Chloride Colorimetric method described by Maiti, (2004) was used to determine the concentration of phosphate ions in the mosquito breeding water. The method is based on phosphate in acidic condition reacting with molybdate to form molybdophosphoric

acid, which is then reduced by stannous chloride to form a blue colour complex. The intensity of the colour, measured by spectrophotometer is directly proportional to the concentration of phosphate present in the sample.

Firstly, Ammonium molybdate solution was prepared by dissolving 2.5g of ammonium molybdate in 17.5 ml of distilled water followed by 28 ml of Conc. H_2SO_4. The solution was made up to 100 ml with distilled water. Stannous chloride solution was prepared by dissolving 2.5g of fresh $SnCl_2$ in 100 ml of glycerol. This solution was then heated in a water bath and stirred with a glass rod to hasten dissolution. Saperately, 50 ml of filtered mosquito breeding water sample was first acidified by a dropwise addition of concentrated H_2SO_4 before the addition of 4 ml of the molybdate reagent and mixing thoroughly. Then, 0.5 ml of stannous chloride was added and the solution allowed to stand for 10 min before measuring the absorbance with a UV-visible spectrophotometer (Jenway 6305 model, Bibby Scientific Ltd. Staffordshire, UK) at 690nm using distilled water as blank. The same procedure was carried out for five phosphate standards of 5 mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml and 25 mg/ml to establish a standard calibration curve of abosorbance vs concentration of the standard phosphate solutions. The concentration of phosphate in each test sample was estimated off the standard calibration curve.

Calculation: mg/L of PO_4^{2-} in the sample = $\frac{\text{mg } PO_4^{2-} \text{ (in 54.5ml final volume) x 1000}}{\text{mL of sample}}$

2.2.6 Determination of nitrite ion concentration

The sulphanilamide-N-(1-naphthyl)-ethylenediamine dihydrochloride (NED dihydrochloride) colorimetric method described by Maiti (2004) was used. The principle is that, under acidic condition (pH 2-2.5), nitrite ion (NO_2^-) reacts with sulphanilamide to form a diazonium salt, which then combines with N-(1-naphthyl)-ethylenediamine dihydrochloride (NED

dihydrochloride) to form a bright coloured pinkish-red azo-dye. The colour produced is directly proportional to the concentration of nitrite ion in the sample.

Firstly, the colour reagent was prepared by mixing 800 ml of distilled water with 100 ml of 85% phosphoric acid and 10g sulphanilamide. After mixing, the volume was made up to 1 L. Standard nitrite ion concentrations ranging between 0.02-0.30 mg/L were prepared from a stock sodium nitrite solution.

A filtered 50 ml volume of the mosquito breeding water sample was mixed with 2 ml of the colour reagent in a 100 ml volumetric flask. The solution was mixed well and the volume adjusted to 100 ml mark with distilled water. The final pH was checked and confirmed to be between 2-2.5. The solution was left to stand for 10 min after which the absorbance was taken at 543nm (Jenway 6305 model, Bibby Scientific Ltd. Staffordshire, UK). The same procedure was used for blank (using distilled water) and standards (using the prepared standard nitrite solutions) and a standard calibration curve created for the standard solutions. The concentration of nitrite in the mosquito breeding water samples were estimated from the calibration curve.

Calculation: Nitrite ion (mg/L) = $\dfrac{\text{mg/L } NO_2^- \text{ from standard curve x dilution factor}}{\text{mL of sample}}$

2.2.7 Determination of nitrate ion concentration

The principle of this colorimetric method for nitrate determination described by Maiti (2004) is that nitrate react with phenol disulphonic acid to produce a nitro-derivative which in alkaline medium develops yellow colour due to rearrangement of its structure. The yellow colour follows Beer's Lambert law and is proportional to the concentration of nitrate present in the sample. There is however interference from nitrite and chloride ions, which have to be removed before analysis. The nitrite and chloride ions were removed by adding sulfamic acid

and silver sulphate solution respectively. Often, the collected mosquito breeding water samples had to be pretreated to remove intense colouration by adding 3 ml of aluminium hydroxide to 150 ml of the water sample. After pre-treatment to remove colour and interfering substances, the mosquito breeding water sample was neutralized to pH 7.0. Then, 50 ml of the sample was evaporated to dryness on water bath. The occuring residue was then redissolved in 2 ml phenol disilfonic acid (PDA) reagent, diluted to the original volume (50 ml) and transferred into Nessler's tube and then 10 ml of 12N KOH and EDTA were added. The solution was finally filtered and diluted to 100 ml with distilled water. The colour developed was read at 410nm using spectrophotometer (Jenway 6305 model, Bibby Scientific Ltd. Staffordshire, UK). The concentration of nitrate (mg/L) in the test mosquito breeding water sample was estimated directly from a calibration curve developed using various concentrations of standard nitrate solutions treated in the same manner as the test sample.

Calculation: NO_3^- (mg/L) = $\frac{\text{mg/L reading from standard curve}}{\text{Volume of sample}}$

2.2.8 Determination of carbon content (Total organic carbon)

Carbon content, measured as total organic carbon in the mosquito breeding water samples was determined using the HATH LANGE TOC CUVETTE-TEST (LCK385 series, Salford United Kingdom) following manufacturer's instructions. The principle of this method (See Appendix for complete operational manual) is that after expulsion of total inorganic carbon (TIC) from a water sample, the total organic carbon is converted to CO_2. The CO_2 is then passed through a membrane into an indicator cuvvete where it causes a colour change that can be evaluated by a photometer. Briefly, 2 ml of the mosquito breeding water samples were pipetted into the digestion cuvete and inserted into the TOC-X5 shaker to expel the TIC. After 5 min, an acoustic signal was emitted signalling the expulsion of TIC. The test sample

was then digested for 2 h at 100°C inside the thermostat with the blue indicator cuvvete upward. It was then cooled down to room temperature and the concentration of TOC read from the DR500 photometer. The value of carbon content was expressed as mg/L.

2.2.9 Determination of Oil and Grease

The liquid-liquid extraction method described by Maiti (2004) was used for the determination of oil and grease in the mosquito breeding water samples. Briefly, 250 ml of the water sample were transferred into a 500 ml beaker and acidified by adding concentrated HCL until the pH was maintained at 2. The acidified sample was transferred into a separating funnel and 30 ml of petroleum ether were added followed by vigorous shaking for 5 min. After clear separation, the lower aqueous layer (water portion) was discarded. The solution was then filtered into a weighted beaker marked W_1 and the solvent completely evaporated on a water bath and then the beaker was weighed again (W_2).

Calculation: Oil and grease (mg/L) = $\frac{(W_2-W_1) \times 1000}{\text{mL of sample}}$

2.3 Prepation of Mosquito Homogenate

Mosquito samples, about 20 each of the larvae, pupae and adult, were homogenised in ice-cold phosphate buffer (0.1M; pH7.2) in 1.5 ml microfuge tubes with Pellet Pestle Motor (Kontes Anachem, Mettler Toledo, Luton, Bedfordshire, UK). The homogenization was carried out on ice and the homogenates were centrifuged for 1 min in a refrigerated centrifuge (Eppendorf Centrifuge 5417R, Motor Park Way, New York, United States) and the supernatants used for protein and detoxification enzymes assays assays. All the mosquito larvae used were of 4^{th} instar, roughly of the same size and all the adult mosquitoes were one day old.

2.4. Protein Determination Assay

Protein cencentration of each homogenate was determined by using Bradford Reagent in a 96 well plates following manufacturer's instructions.

2.5 Biochemical Enzymes Assays

Assay of the three major detoxification enzymes, cytochrome P450 (P450), glutathione transferase (GST) and α & β-Esterases, were carried out using procedures outlined by WHO (1998) and by Poupardin *et al.*, (2008) with modifications. The principles of these assays is based on the ability of these enzymes to conjugate specific substrates ($3,3^1,5,5^1$-tetramethyl benzidine for p450; 1-chloro-2,4-dinitrobenzene for GST and Naphthyl acetates for the non specific esterases) into a coloured products which absorbs light at specific wavelengths.

2.5.1 P450 Activity Assay

Twenty (20) µL mosquito homogenate were mixed with 80 µL of potassium phosphate buffer in a microtitre plate well and 200 µL of the working solution (5 ml methanol solution of 0.002 mg/ml of $3,3^1,5,5^1$-tetramethyl benzidine in 15 ml of 0.25M sodium acetate buffer; pH 5.0) was added. Finally, 25 µL of 3% hydrogen peroxide was added to the well. The mixture was incubated at room temperature for 2 h and the absorbance was read at 650nm using a microplate reader (Modulus Microplate Reader; Turner Biosystems Sunnyvale, California, United States). Control and calibration standards (varying concentrations of standard cytochrome C) were treated similarly and all assays were performed in triplicates.

Calculation: Standard calibration curve of absorbance at 650nm of varying concentrations of cytochrome C was prepared. The P450 activity in the test sample was estimated from readings off the calibration curve. The specific activity of P450 calculated in µmol per mg protein was then calculated as follows:

$$\text{P450 Activity (µmols p450/mg protein)} = \frac{\text{Readings from the calibration curve}}{\text{mg of total protein}}$$

2.5.2 GST Assay

10 µL mosquito homogenate were mixed with 200 µL of GSH/CDNB working solution (125 µL of 63 mM CDNB in 2.5 ml of 10 mM GSH solution) in a microtitre plate well. The reaction was read immediately at 340nm as a kinetic assay for 5 min. Blanks were prepared with 10 µL of the phosphate buffer mixed with 200 µL of the GSH/CDNB working solution and all the assays were performed in triplicates.

Calculation: The conjugation of CDNB with GSH produced 3-(2-chloro-4-nitrophenyl)-glutathione. The reaction obeys Beer Lambert law and the extinction coefficient at 340nm has been determined as $5.76mM^{-1}$ (correcting for a path length of 0.6cm) (Perera *et al.*, 2008, WHO. 1998). Therefore, the rate of the reaction was determined from the kinetic curve of absorbance against time and GST activity (the concentration of the product formed per minute) was calculated from the equation:

$A=\varepsilon cl$

Where A= Absorbance at 340nm

ε = Extinction coefficient

c = concentration and

l= pathlenth.

GST specific activity (correcting for mg of total protein) was calculated as CDNB conjugated in µmol product formed min^{-1} mg^{-1} protein.

2.5.3 α and β-esterase Assay

Twenty (20) µL mosquito homogenate were mixed with 200 µL of 1-Naphthyl working solution (1 ml of 30 mM 1-Naphthyl acetate mixed with 99 ml of potassium phosphate buffer; pH 7.2) and 2-Naphthyl acetate working solution (1 ml of 30 mM 2-Naphthyl acetate mixed with 99 ml of potassium phosphate buffer; pH 7.2) in seperate microtitre plate wells for α and β-esterases assay respectively and incubate for 15 minutes at room temperature. 50 µL of fast blue B stain solution was then added to the wells. A separate blank was set up for each of the two esterases containing 20 µL of potassium phosphate buffer also mixed with 200 µL of the working solutions and 50 µL of stain solution. The mixture was read at 570nm as an end point assay using a microplate reader (Modulus Microplate Reader; Turner Biosystems Sunnyvale, California, United States). All the assays were performed in triplicates.

Calculation: The products of the 1-Naphthyl acetate (1-NA) and 2-Naphthyl acetate (2-NA) are 1-Naphthol and 2-Naphthol respectively. Standard calibration curves were prepared for the two chemicals with varying concentrations in 0.02M phosphate buffer pH 7.2. Absorbance values obtained from the mosquito samples were then converted to µmols 1-Naphthol for the α-esterase and to µmols 2-Naphthol for the β-esterase from the relevant standard curves. The specific activities of the two esterases were calculated as:

$$\text{(a) } \alpha\text{- esterase } (\mu\text{mols 1-Naphthol min}^{-1}) = \frac{\mu\text{mols 1-Naphthol from calibration curve}}{\text{15 minutes (period of incubation)}}$$

Then, specific α-esterase activity per mg protein expressed in µmols1-Naphthol min^{-1} mg^{-1} was calculated by dividing the value in 'a' above by the mg total protein. The same steps

were carried out for β-esterase activity using μmols 2-Naphthol in this case instead of 1-naphthol.

2.6 Determination of Reduced (GSH), Oxidized (GSSG) and Total glutathione (tGSH).

Reduced, oxidized and total glutathionelevels in the mosquito samples were determined using Glutathione assay kit (NWLSS^{TM} NWK GSH01, Northwest Life Science Specialities, LLC Vancouver, WA 98683). The assay was conducted on the three life stages of *Anopheles gambiae* (larvae, pupae and adult) following manufacturer's instructions and procedures (See Appendix for the Manufacturer's protocol).

2.7 *Anopheles gambiae* Complex Polymerase Chain Reaction (PCR) and Species Identification

Anopheles gambiae intraspecie identification was carried out by the standard PCR procedure described by Scott *et al.*, (1993); courtesy of the Nigerian Institute of Medical Research, Yaba, Lagos-Nigeria.

2.8 Statistical Methods

The SPSS v.20 statistical package (IBM SPSS Software, United Kingdom) tools were employed to carry out various statistical analyses. Firstly, one way ANOVA was used to investigate differences in mean distribution of the physico-chemical environmental factors, larval densities, enzyme activities, and the various forms of glutathione across the three study zones. Secondly, Mixed Linear Model's Bonferoni Post-hoc pairwise comparism tests were used to examine the effect of each of the study zone on the mean distribution of the aforementioned environmental, enzymatic, and glutathione factors. Consequently, Zone A against B, A against C and B against C were compared for significance in mean distribution of each environmental, enzyme and glutathione factors. Then, Linear Regression analysis was

carried out to investigate correlations or associations between the physico-chemical environmental factors and *An. gambiae* larval densities, between physico-chemical environmental factors and the detoxification enzymes activities and between the detoxification enzymes activities and the levels of the three forms of glutathione. Furthermore, Factor analysis was employed to extracts the principal components from the physico-chemical environmental factors, the detoxification enzyme activities as well as the three forms of glutathione studied across the three life stages of *An. gambiae*. Then, Regression in Principal component was used to investigate the combination of the physico-chemical environmental factors with the most significant combined effect on larval density. Finally, Redundancy Analysis was carried out to investigate the combined effect of the extracted principal components from the physico-chemical environmental factors on those of the detoxificatiion enzymes activities. The same analysis was used to examine the association between a combination of the extracted glutathione components and the detoxification enzyme activities.

CHAPTER THREE

3.0 Field Study Report

3.1 Introduction

This PhD study is aimed at assessing the role of physico-chemical environmental factors arising from some human related activities; notably intensive agriculture and the processing, usage and discharge of petrochemical products in the environment, as sources of selection pressure for the development and emergence of insecticide resistance in *An. gambiae* in Nigeria. Consequently, identifying Anopheles breeding ecologies where these human related activities serve as sources of discharge of environmental xenobiotics into mosquito breeding sites in Northern Nigeria, was the first major objective of this research. Thus, studies in several metropolitan areas; towns and villages located in Kano and Jigawa States; Northern Nigeria were conducted. The field studies involved first, prospecting for, and identifying active Anopheles breeding sites in and/or around areas where these human related activities are taking place. Then carrying out larval sampling and rearing as well as analysing the water quality at the breeding sites. Furthermore, similar studies and analyses were carried out in domestic or residential settings. Kano and Jigawa states in Northern Nigeria were chosen because of their close proximity (the two states actually used to be one before it was split into two) and their interrelatedness and linkages in terms of culture, politics, commercial, industrial and agricultural activities, as well as similar geographical settings. The north of Nigeria where these two states are located accounts for more than 60% of Nigerian population, and moreover, the two states (Kano and Jigawa) constitute more than 30% of this norther Nigerian population (NPC, 2006b). Importantly, the region has the highest malaria burden relative to Southern Nigeria. Also, more than 65% of Nigeria arable land is located in the north (NPC, 2006a), thus intensive agriculture is the major economic activities in this region. Although, crude oil exploration takes place in parts of the south (Niger-Delta),

processing, use and discharge of refined petroleum and other hydrocarbon products is highest in the North due the larger population. Therefore, contamination of mosquito breeding sites by agricultural and petrochemical products is expected to be high in this region relative to the south. This scenario is exacerbated by poor infrastructure in this region which precipitates the creation of abundant mosquito breeding sites.

For convenience and clarity, the areas visited and the sites sampled during the field exercise is tabulated below (Table 3.1).

Table 3.1 List and description of the field Study areas

Study zone	Nature and characteristic of study zones	No. of sites in each zone
Zone A	Areas of intensive agriculture involving the use of pesticides, fertilizers and other agro-allied chemicals	3
Zone B	Residential or domestic environments	4
Zone C	Commercial centres involving the sale, processing, refinements and discharge of petrochemical or hydrocarbon products.	3

An. gambiae larvae were collected in all the breeding sites visited across the three study zones and levels of physical environmental factors, including pH, temperature, conductivity, transparency, total dissolved solids (TDS), and dissolved oxygen (DO) in the *Anopheles* breeding water were determined during the sampling exercise. Water samples from all the visited Anopheles breeding sites were also collected and used in the laboratory to assess the levels or concentration of some chemical environmental factors including biological oxygen demand (BOD), sulphates, phosphates, nitrites, nitrates, carbon content and oil and grease.

The sampled larvae were kept in thier breeding water and transported to the insectory. Some of the larvae were then taken out for storage and some were reared until they emerged into pupae and adult before storage. All the stored samples (larvae, pupae and adults) were finally transported to the United Kingdom for further biochemical and other analyses. The major studies conducted during the field exercise, and the results and observations recorded are detailed below.

3.2 Study Sites

This study was conducted in several towns and villages located across Kano and Jigawa states of Northern Nigeria. Kano is situated in Northwest Nigeria between Latitudes 11° 30′N and Longitudes 8° 30′E. Kano state borders the states of Katsina to the west, Jigawa to the East, Bauchi to the South-east Kaduna to the South-west and Niger Republic to the North. It has a population of about 13 million people, second only to Lagos state in Southwest Nigeria, and a land mass of approximately 18,684 square km. In addition, Kano has a population density of approximately 470/km^2. Kano city is located 481 m above sea level (John, 2007). The state's mean annual rainfall ranges between 800 mm in the extreme north of the state to 1000 mm in the extreme south. The rains usually last for three to four months (June-September). There are usually four seasons within the state; a dry-cool season (November-February) marked by cool dry weather with occasional haze and dust and with average low temperature of between 11°C to -1°C; a hot-dry season (March-May) marked by a very hot dry weather with temperature reaching up to 44°C; the wet-warm season known in local dialect as 'Damuna' (June-October) is the proper wet season; and lastly a dry warm season (Mid October to Mid November) marked by high humidity and temperature. The region features predominantly savannah vegetation with a hot semi arid climate to the extreme north. The average precipitation per year is 690 mm, the bulk of which falls between July and September (NIMET, 2012)

Jigawa state is also situated in the northwest part of Nigeria between Latitudes 11.00°N to 13.00°N and Longitudes 8.00°E to 10.15°E. Jigawa state is bordered by the states of Kano and Katsina to the west, Bauchi to the east and Yobe to the northeast. It shares an international border with Zinder region of Republic of Niger to the north. Jigawa has a total land area of approximately 22, 410 km^2 with topography characterised by undulating land with sand dunes of various sizes spanning several kilometres. In the southern part of the state is the basement complex, while the northeast is comprised of sedimentary rocks formed in the Chad basin. The major rivers include Hedeja, Kafin Hausa and Iggi rivers with many tributaries. Greater parts of the state lie within the Sahel savannah with little vegetation cover, but some of the southern parts lie within the guinea savannah. The state has one of the highest FADAMA wetlands in the country (3,437.79 square kilometres). The climate is characterized by two main seasons; the wet rainy season (May-September) brought by the wet breezes from the Atlantic coast of West Africa and the dry Harmattan season (November-April), characterized by dry dusty winds blowing form the Sahara Desert (SEEDS, 2009). Temperature ranges between 42°C in the wet rainy season to 10°C in the dry cold season and the average annual rainfall is less than 600 mm (NIMET, 2012). Irrigation farming supports cultivation of many crops for most parts of the year, especially during the long dry season. Agriculture is the major economic activity, with subventions from the Federal government which constitute the major income (SEEDS, 2009).

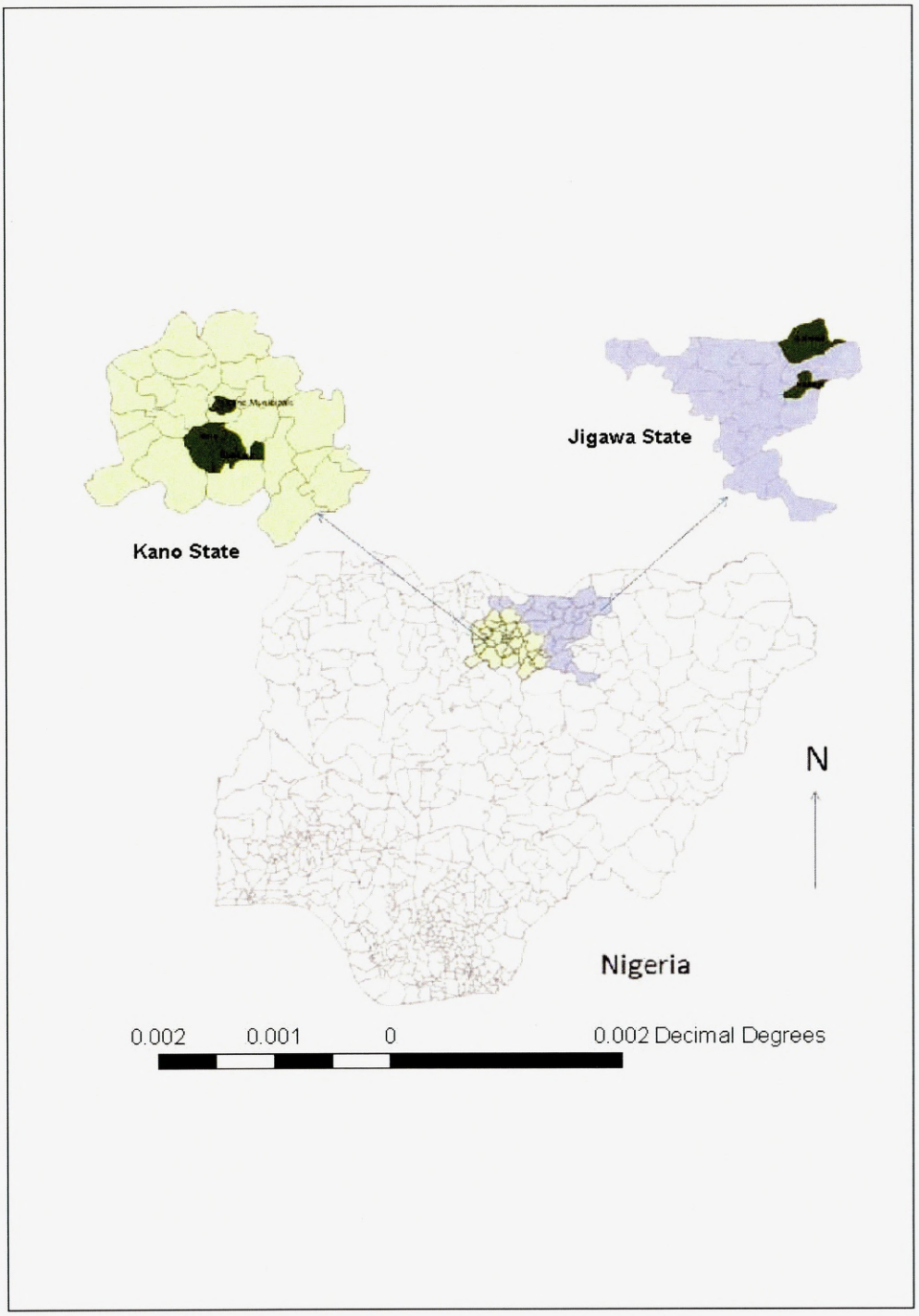

Fig. 3.1 Map of Nigeria projecting the locations of the field study areas in Kano and Jigawa states in Northern Nigeria. The towns and villages where the study was conducted were highlighted in green; while the locations of the two states within the larger Nigeria map were also highlighted in lime and purple (larvrnder).

3.3 Larval Prospecting

Search for potential larval habitats was conducted throughout the entire rainy season of 2011 (June-September), during which the field study was conducted. Prospecting or searching for active larval breeding sites was conducted two to three times a week across the three study zones (Table 3.1). Across these study zones, searching for areas where water stagnates was conducted and upon coming across any stagnant water bodies, a search for the presence of mosquito larvae (Anophelene and other species) was carried out. Larval prospecting was conducted in each of the study zone at least once every week between June and September.

3.4 Identification of Mosquito Species

Upon identifying active breeding habitats in any of the zones visited, identification of mosquito larval species thriving in the habitats was carried out on the bases of morphology and behaviour. As described in previous studies (Merrit *et al.*, 1992; Clemments, 2000), Anopheles mosquito larvae were found to possess a well-developed head with mouth brushes used for feeding, a large thorax and a segmented abdomen. They have no legs. In contrast to other mosquito species (*Culicines*), Anopheles larvae position themselves so that their body is parallel to the surface of the water. This is because unlike other mosquito species, they posses no respiratory siphon and breathe through spiracles located on the 8th abdominal segment and therefore must come to the surface frequently. The larvae spend most of their time feeding on algae, bacteria, and other microorganisms in the surface microlayer. They dive below the surface only whenever disturbed. Larvae swim either by jerky movements of the entire body or through propulsion with the mouth brushes. In contrast, Culex mosquitoes were found to hang themselves longitudinally with the head hanging downward below the air-water interface and are less active even when disturbed (Paaijmans, 2008). After sampling and rearing of the *An. gambaie* to adulthood, molecular (PCR) species identification was carried out using protocols described by Scott *et al.,* (1993) (See Material and Methods) to

identify members of the *An. gambiae* species complex. Almost all the Anopheles mosquito sampled during the field work and subsequently used to carry out the biochemical and glutathione analyses belong to the members of *An. gambiae s.s* (See Section 3.8).

3.5 Larval Sampling, sorting and Determination of Larval Density

Sampling of mosquito larvae from breeding sites identified in each of the three study zones was conducted at least once a week throughout the field study period (June-September, 2011). Stagnant water bodies within and/or around farmlands, residential areas and sites of petrochemical commercial activities were surveyed for the presence of mosquito larvae. Larval collections were made using 350 ml standard mosquito dipper as described by Service (1993). Several dips were made depending on the size of the breeding site. After each larval collection, the larvae were sorted first on the basis of species into Culicines and Anophelines. The stage of the larval development i.e. 1^{st}, 2^{nd}, 3^{rd} and 4^{th} instars was noted. All members of the Culicine species were returned back to the breeding habitat after sorting and counting while the Anopheles larvae were collected in the same breeding water after counting, in light coloured plastic containers. Estimates of the quantity of the breeding water were made and larval densities were determined and expressed as number of larvae per litre of breeding water. After larval sorting and counting, the Anopheles larvae collected were immediately taken to the insectory for rearing. Pictures were taken to describe the scenery around the mosquito breeding habitats and also to show sampling workers at work (See appendix).

3.6 Mosquito Rearing, Storage and Transport

In the insectory, any 4^{th} instar larvae present was collected in 1.5 ml Ependoff and immediately stored in -80 freezers. The remaining larvae were reared in the insectory until they emerged into pupa and adult. Rearing of the Anopheles larvae was carried out following standard protocol (Gerberg, 1970). The larvae were reared in light plastic containers covered

with fine mesh mosquito nets. They were reared in the same water from which they were sampled to maintain the impact of the chemical environmental xenobiotics present in the breeding habitat. The 4th instar larvae and pupae were transferred alive directly from the breeding container into 1.5 ml Eppendorf tubes as they emerged. Some of the Eppendorf tubes contained RNA later (RLT) buffer (Qiagen) to protect the integrity of the genetic materials (genomic DNA and total RNA). One day old adults were also collected as they emerged from the breeding water. They were first transferred into cages from the breeding containers and the cages containing the adult mosquitoes inserted into -20 freezers for few minutes until the adult mosquitoes were knocked unconscious by the freezing environment. They were then individually picked, while still inside the freezer, and transferred into the 1.5 ml storage tubes that were appropriately labelled to reflect the life stages, sites of sampling and study zone. The samples were kept in -80 freezers until the day of transport to the UK. They were transported to the University of Abertay preserved on ice in temperature regulating containers. Upon arrival at the UK, the samples still on ice were immediately transferred again into -80 freezers until laboratory analyses.

3.7 Verbal Interviews

A short verbal interview was conducted with farmers during mosquito sampling in breeding sites located within and/or around intensive agricultural areas. The farmers were asked to provide information on the types of fertilizers they use on their farms, the kind of pesticides they applied to control agricultural pest and the frequency of application. More than two farmers were interviewed in each of the three breeding sites visited across this study zone and their responses provided information (See section 3.8) on types and nature of agro-chemicals and fertilizers commonly applied to farmlands located around this study zone.

3.8 Results

3.8.1 Larval Density

In order to examine the role of various human related activities as sources of variations in the levels of the physical and chemical environmental conditions of *An. gambiae* breeding ecologies, and to evaluate the impact of these variations on larval growth, productivity and survival, determination of larval density per litre of breeding water as well as measurements of the levels of physical and chemical environmental factors were carried out. The assessments were carried out in different breeding ecologies identified and grouped into three major study zones (Table 3.1) according to the nature of the major human related activities taking place around the mosquito breeding sites. This approach was informed by increasing reports from other regions implicating human activities with changes in malaria vector population structure (Awolola *et al.*, 2005; Dabire *et al.*, 2008; Mwangangi *et al.*, 2010; Yadouleton *et al.*, 2011; Imbahale *et al.*, 2011). In some of the breeding sites visited, *An. gambiae* was found to be breeding alongside other forms of mosquito (mainly *Culex spp*). Therefore, total mosquito larvae and *An. gambiae* larval count (per litre of breeding water) was carried out to determine the total mosquito larval and *An. gambiae* larval densities.

Table 3.2 Mosquito larval densities within sites and across the three study zones

Sites	Larval Densities(per litre of breeding water)				
	Total Mosquito Larvae	***An. gambiae* Larvae**	***An. gambiae* Larvae (%)**	**Culicines Larvae (%)**	**Study Zone[a]**
Site 1	43	40	90.0	10.0	
Site 2	27	26	96.3	3.7	A
Site 3	29	20	69.0	31.0	
Mean ± SD	33 ± 8.7	28.7 ± 10.3	85.1± 14.3	14.9 ± 14.3	
Site 1	100	100	100	0.0	
Site 2	40	40	100	0.0	
Site 3	107	100	93.4	6.6	B
Site 4	60	60	100	0.0	
Mean ±SD	76.8 ± 32.1	75 ± 30	98.4 ± 3.3	1.65 ± 3.3	
Site 1	26	17	65.4	34.6	
Site 2	34	14	41.2	58.8	
Site 3	23	13	56.5	43.5	C
Mean ± SD	27.7 ± 5.7	14.7 ± 2.1	54.4 ±12.2	45.6 ± 12.2	

[a] Zone A, intensive agriculture; B, residential/domestic; C, petrochemical breeding ecologies

Data in Table 3.1 showed that sites located in residential/domestic environments (zone B) recorded higher larval densities compared to the two other study zones. With the exception of site 2 study zone C, *An. gambiae* constitute the largest percentage of the total larval density in all the sites across the three study zones.

3.8.2 Responses from Farmer's Interview

The result of farmer's interview (Table 3.2) provided information on the chemical nature of the fertilizers and agro-pesticides used in this area.

Table 3.3 Types of fertilizers and agro-pesticides applied to farmlands located around mosquito breeding sites in intensive agricultural areas (Study zone A)

Sites	Fertilizers	Pesticides/Herbicides	Class
Site 1	NPK Fertilizer	Cypermethrin	Pyrethroids
	Organic Manure	Endosulfan	Organochlorines
		Fipronil	Organochlorines
		Carbofuran	Carbamates
Site 2	NPK Fertilizer	Cypermethrin	Pyrethroids
	Organic Manure	Deltamethrin	Pyrethroids
		Diazinon	Organophosphates
		Dimethoate	Organophosphates
Site 3	NPK Fertilizer	Lambdacyhalothrin	Pyrethroids
	Organic Manure	Cypermethrin	Pyrethroids
		Deltamethrin	Pyrethroids
		Methylparathion	Organophosphates
		Carbofuran	Carbamates

3.8.3 PCR Species Identification

In order to confirm the sub species identity of the sampled *Anopheles* mosquitoes, a polymerase chain reaction for mosquito species identification procedure was carried out on the adult members that emerged from the larvae collected from each sites across the three study zones as described by Scott *et al.*, (1993). The PCR experiment was conducted in Nigerian Institute for Medical Research, Yaba-Lagos, Nigeria. The result of the PCR experiment ran on 1% aggarose gel (Fig. 3.2) showed that all the sampled mosquitoes produced a band size corresponding approximately to the 400 bp ladder (Fig. 3.2). Using the universal primer (UN) and *An. gambiae* (GA) specific primer for 28s ribosomal DNA (Scott *et al.*, 1993), the sequence of the 28s ribosomal gene was extracted from National Centre for Biotechnology InformAion (NCBI) as follows:

> Anopheles gambiae 28s ribosomal PCR product

GTGTGCCCCTTCCTCGATGTGCGCAAGCTCGTCTTGGTCTGGGGACCACGTCGA
CACAGGGGATACTTTTGTGAGAGCAAGAGTGTACTTAGTTGAGTGTAGCAAGGG
ATCGCGTGCCCCTTCCTCGATGGCGTAACGAACCATCTTGGTCTGGGGACCGTGG

TACCGTGCTCTGGTGAAGCTTGGTGCGTGCTCTTTCCTTGTCAGACGAGTGACTT
GACTTGGTCTGGAGACCGTTCCTTAACACTAGTGGACAAGAGCTGGCTACTTCCG
TGTCAGACGAGTGACTTGACACGGTATGGAGCGGAACACGTAAACACTAGTGAC
TTGTCGGCGTGCCTCGTTCTCGACTTGATTGTCTTGATGTGAG
AAACGTGCCGACCAAACCAG

Expected PCR product size for GA species =391bp

Universal primer (UN) **GTGTGCCCCTTCCTCGATGT**

GA specific primer **CTGGTTTGGTCGGCACGTTT**

GA reverse complement **AAACGTGCCGACCAAACCAG**

Therefore, from this information, the expected size of the *An. gambiae* PCR product is exactly 391bp. From the PCR product (Fig. 3.2), Marker lane 1 is DNA ladder where 500bp is the brightest band. The *An. Gambiae* PCR product bands appears to be in approximate line with the 400bp ladder band, confirming that all 20 DNA samples analysed are very likely to be from *An. Gambiae.* and not from another mosquito species.

Previous studies (Boreham *et al.*, 1979; Molineaux and Gramiccia, 1980; Hanney, 1960; Service, 1965; Rishikesh, Dideco, Petraca and Coluzzi, 1985) have already established members of this *An. gambiae* complex as the major vectors of malaria in Northern Nigeria with members of *An. funestus* playing a minor role ((Gills and Coetzee, 1987). So it is not surprising that all the *Anopheles* mosquito samples collected during this study belong to this species complex.

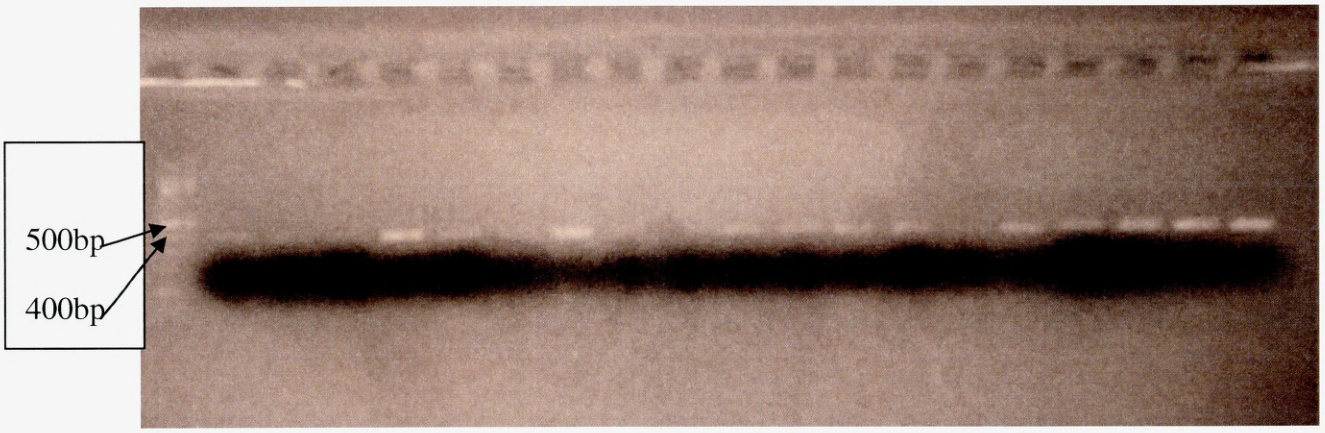

Fig. 3.2 DNA bands produced by ribosomal DNA-polymerase chain reaction amplification of DNA. Mosquito samples from all the ten breeding sites visited across the three study zones were subjected to *An. gambiae* specie identification. Lane 1 contain 1kb Ladder size standard while lane 2-20 contained mosquito DNA samples from the ten breeding sites visited, all of which were identified as members of *Anopheles gambiae s.s.* as previously described by Scott *et al.*, (1993).

3.9.1 Discussion

The results and observations recorded during this field exercise showed that mosquito species can breed in different ecological conditions irrespective of the human related activities taking place within and/or around the breeding sites. Active Anopheles mosquito breeding sites were located in habitats which displayed marked differences in physical nature and xenobiotic characteristics. In most of the breeding environments visited, Anopheles species were found to breed exclusively alone. Only in a few sites that Culicines were found to breed alongside the Anopheline, though usually in lesser population compared to the Anopheles species. It was observed that some of the breeding water bodies where sampling was conducted appeared relatively clean and little contaminated, while others were dirty, muddy, murky, and contaminated with various materials. Previous studies (Sattler *et al.*, 2005) have suggested that Anopheles mosquito prefer relatively clean and less contaminated water bodies for breeding. While this observation still holds for many of the breeding sites visited, especially in the domestic/residential environments (study zone B), the nature of some of the

visited breeding habitats were in sharp contrast to this observation. Anopheles and Culex species were collected from dirty, muddy, fowl smelling and obviously contaminated water bodies. Close examination of the breeding habitats and the surrounding environs showed that most of the contaminants were washed into the mosquito breeding sites from the surrounding environments. Most of the breeding sites visited in intensive agricultural environments as well as commercial urban centres, where petrochemical products were sold, used, refined, processed and/or discharged (study zones A and C), were of this nature. The nature and degree of contamination of these breeding sites were assessed by measuring the levels of various physical and chemical environmental factors (See Chapter 4).

The results of larval densities (Table 3.2) showed that domestic/residential environments (study zone B), whose breeding habitats were relatively clean and little contaminated as compared to those in study zones A and B, recorded the highest *An. gambiae* larval densities. This agrees with the observation of Sattler *et al.*, (2005) which suggests that Anopheles mosquitoes prefer clean and little contaminated water bodies. However, the presence of large amount of *An. gambiae* larvae from breeding sites located in intensive agriculture and petrochemical zones, which showed marked differences both in physical nature as well as levels of environmental xenobiotics (See Chapter 4), showed that Anopheles mosquito may be showing some levels of adaptations to various chemical pollutants and contaminants present in the environments. Adaptations to environmental conditions is not limited to mosquito or insects alone, it is a general and a conserved phenomenon observed in all living organisms from prokaryotes to eukaryotes (Evelyn, 1965; Ernst, 1982). Insects in particular, whose life history is distributed between two habitats (aquatic and terrestrial), have been reported to display functional and dynamic adaptation leading to changes in both tolerance and behaviour (Tauber *et al.*, 1986). Of particular interest is the observed presence of *An. gambiae* larvae in breeding sites laden with petrochemical products. Historically, products

from petrochemical and hydrocarbon compounds (kerosene, premium motor spirit, spent engine oil etc) have been used to control mosquito larvae population. This control strategy has been relatively effective in reducing mosquito larval abundance in many parts of the world (Burton, 1967; Rozendaal, 1997)). However, the result of this study, which showed the presence of mosquito larvae in breeding sites laden with petrochemical and hydrocarbon compounds, indicates that mosquito larvae may have developed tolerance to these compounds in Nigeria. This can be proved by displaying higher detoxification enzymes activities in mosquito samples from petrochemical laden breeding sites relative to non petrochemical laden sites.

Large amount of mosquito larvae, mostly Anopheles species, were sampled from puddles, flooded furrows, shallow wells, animal hoof holes, and ponds within and/or around intensive irrigated farmlands. Most of the farmers in Nigeria were known to use different agro-chemicals including pesticides and fertilizers to control agricultural pests and enrich soil fertility. Indeed, most farmers interviewed during this field study confirmed that they constantly apply these chemicals and fertilizers to their farms. Their responses (Table 3.3) showed the type of fertilizers and agro-pesticides used and thier classes. Chemical categorization analysis of these agro-pesticides showed that they share similar structures and activity relationships with many of the chemical synthetic insecticides commonly used in mosquito control. They also belong to the same chemical classes with these insecticides. Most synthetic insecticides and pesticides belong to four major chemical classes which include; Organochlorines, Organophosphates, Carbamates and Pyrethroids (Kumar, 1984). Residues from these agro-pesticides are washed by rain into the surrounding mosquito breeding sites. This was established by measuring the levels of various chemical species which forms part of the chemical structure of these pesticides. Results (Chapter 4) showed that these chemical species were detected in significant higher quantities from breeding sites

located around the intensive agricultural areas (study zone A) compared to the two other study zones. Larval densities (Table 3.1) from the breeding sites in this zone as well as petrochemical laden zone (study zone C) were also lower compared to those recorded for domestic/residential areas.

3.9.2 Conclusion

The results and observations recorded during this field exercise suggest a functional and dynamic shift in behaviour and relationship between Anopheles mosquito and its ecological environments. *An. gambiae* larvae were collected in breeding habitats containing relatively high amount of chemical species hitherto used as agents of its control. Although, this study revealed marked differences in physical nature and xenobiotic characteristics of different *An. gambiae* breeding ecologies, assessing the implication of this observation is necessary. For instance, investigating the relationships between the levels of the determined environmental physical and chemical factors and larval densities is necessary in order to understand their effect on larval growth, development and survival. This kind of study could also be used to understand factors affecting habitat productivity. It is also necessary to subject the sampled *An. gambiae* mosquitoes (the three life stages) to biochemical and molecular analyses in order to determine their response to various detoxification mechanisms. This kind of study could be use to establish the activities of the breeding habitats in this study, and the human related activities taking place around them, as a source of selection pressure for the potential development and emergence of insecticides resistance in the mosquito samples

CHAPTER FOUR

4.0 Effect of Physico-chemical Environmental Factors on *Anopheles gambiae* Larval Density

4.1 Impact of Physical and Chemical Factors on Mosquito Growth and Survival

Temperature is among the various physical factors that can affect larval growth and development. Previous studies have reported temperature to influence insect's growth rate. This effect is thought to be due to increase in enzyme-catalysed growth promoting reactions at higher temperatures (Atkinson, 1994). There is, however, a point at which further increase in temperature will alter the structure and stability of these enzymes and thereby destroy their activity. This may lead to the death of the larvae. Insects in general have various ways by which they respond to unfavourable temperature. For example, they can change their behaviour and development by producing heat shock proteins to increase their tolerance to high temperature (Neven, 2000). Furthermore, responses to high or low temperature differ between larval species and even between larval instars. Younger insects' larvae seem to tolerate high temperature than older ones. Degree of temperature and length of exposure also affect larval mortality (Love and Whelchel, 1957). A study on *Aedes triseriatus* larvae reared at 25°C showed that they did not develop into adult when the temperature was increased to 38°C. The same study showed that larvae stop developing at 6-8°C but resume development when the temperature was increased to 25°C (Jalil, 1972).

Studies on *An.. gambiae s.l.* response to changes in temperature reveals information not very different from those of other related mosquito species. *An. gambiae* larvae breeds in small open areas and are therefore under direct influence of the temperature of their surroundings. A study by Depinay *et al.*, (2004) consisting of many field and laboratorydata collected on the response of *An. gambiae* to temperature variations showed the growth and development rate of *An. gambiae* to increase with increasing temperature. Their development peaks at

about 37°C, after which it sharply decreases. Another study documented high larval development in breeding water temperature of above 37°C in the tropics, though there is constant temperature variation especially during the raining seasons (Munga *et al.*, 2005). Bayor and Lindsay (2003) reported reduced larval development at temperatures above 32°C and below 18°C. A generalised conclusion on the effect of temperature on larval growth and survival is difficult due to a constant temperature variation. In general, many extrapolations have been made with data obtained from laboratory studies where *An. gambiae* larvae were exposed to varying degree of constant temperatures. Whether observations from these kinds of studies can be applied to larvae *in situ* is a matter for debate. What is clear from various studies is that temperatures affect larval growth and survival and could therefore be an important physical factor affecting larval density.

Transparency or turbidity is a function of total dissolved solids, and some studies have reported larval population dynamics in breeding sites of varying turbidity. Available data showed that some mosquito species prefer turbid breeding sites, while others breed well in clearer sites. *An. gambiae* was found to colonise a clearer clean water bodies (Sattler *et al.*, 2005), while other studies reported active breeding sites for *An. gambiae* in highly turbid waters (Mwangangia *et al.*, 2007). Irrespective of these differing opinions, none of the reported studies have to my knowledge given a clear relationship between turbidity, as a function of total dissolved solids and larval growth and survival and the consequent effect on larval density. The pH and electrical conductivity are functions of ionic content of a medium. pH of a water body has also been found to increase with increasing temperature due to changed concentration of carbondioxide (Ademoroti, 2003). It is difficult to investigate the effect of pH and electrical conductivity as isolated factors on growth and development of aquatic organisms, because their levels usually depend on the presence of other physical and chemical parameters. Dissolved Oxygen (DO) and Biological Oxygen Demand (BOD) are

two abiotic factors whose levels depend on the presence and activities of other biotic as well as abiotic factors in an aquatic environment. Factors such as total dissolved solids, presence of certain chemicals, decaying organic matter, and the nature and population of microorganisms among other things, affect the levels of DO and BOD of a given breeding site. To my knowledge, interactions of many of these physical factors and how they affect *An. gambiae* larval density has not been largely investigated.

The chemical nature of a mosquito breeding site depends largely on the nature and type of human activities taking place in the surrounding environments. Breeding sites found within and/or around intensive agricultural areas are expected to be contaminated by agro-allied chemicals such as pesticides and fertilizers; in general the specific chemical contaminants present would depend on the type of chemicals and fertilizers being applied. Chemical species such as nitrates, nitrites, sulphates, phosphates and carbon containing compounds can be found in breeding sites where phosphate and nitrate-based fertilizers are used. Various pesticides as well as animal droppings especially cow dung (usually used as an organic fertilizer) could also contribute to the presence of these chemicals species. Furthermore, carbon content and oil and grease will be found in high amount in breeding sites located in areas where petrochemicals/hydrocarbon are constantly processed, used and/or discharged. Since mosquito larvae have been reported in oil and grease contaminated environments (Sattler *et al.,* 2005) and given that the application of oil and grease and other hydrocarbon compounds to control mosquito larvae has been a practice in many parts of the world for decades, it is relevant to investigate the implication of the presence of these chemicals in mosquito breeding sites on larval growth and development and their effect if any, on larval density. Many studies have looked at the overall relationship between various agricultural practices and incidences of insecticides resistance in malaria vector. The general consensus is that practices such as use of pesticides could serve as selection pressure for resistance to

public health insecticides. However, the specific mechanisms responsible for this effect is still poorly understood, and further research is therefore needed to investigate the effect of specific chemical contaminants arising from various agricultural practices, not only pesticides application but also use of both natural and synthetic fertilizers on mosquito growth, development and behaviour across the three life stages of mosquito. In this chapter, the effect of some physical and chemicals environmental factors on *An. gambiae* larval density will be evaluated.

4.2 Introduction to factor and redudndancy analysis

Factor analysis is a statistical method used to describe variability among observed, correlated variables in terms of potentially lower number of unobserved variables called factors or principal components (PCs). In order words, it is possible for example, that variations in three or four observed variables mainly reflect the variations in fewer unobserved variables. The major application of factors analysis are to reduce the number of variables and to detect structure in the relationships between variables (Barthelomew *et al.*, 2008). Factor analysis searches for joint variations in response to unobserved latent variables. The observed variables are modelled as linear combinations of the potential factors plus error terms. The information obtained about the interdependences between observed variables can be used later to reduce the set of variables in a dataset. Factor analysis technique is equivalent to low rank approximation of matrix of observed variables. Factor analysis originated in psychometric, and is used behavioural ciences, physical sciences, social sciences and natural sciences (Bartholomew, *et al.*, 2008).

There are different type of factor analyssis. These include; principal component analysis (PCA), which is widely used to extract factors or PCs, Canonical factor analysis, Common

factor analysis, Image factoring, Alpha factoring, and Factor regression model. Some common terminology used in factor analysis include:

(1) Factor loading:- These are correlation coefficients between the variables (rows) and factors (columns). It is the percent of variance in the indicator variable expalined by the factor.

(2) Eigenvalues:- The eigenvalues for a given factor measures the variance in all the variables which is accounted for by that factor. The ration of eigenvalues is the ratiom of explanatory importance of the factor with respect to the variables.

(3) Factor scores:- These are the scores of each case (row) on each factor (column).Computing factor scores allows one to look for factor outliers.

(4) Scree plot:- The Cattell sree test plots the components as the X axis and the corresponding eigenvalues as the Y axis. As one moves to the right towards the components, the eigenvalues drop. When the drop ceases and the curve make an elbow towards less steep decline, Cattell's scree test says to drop all further components after the one starting the elbow.

(5) Varimax rotation:- This is an orthogonal rotation of the factor or components axes to maximize the variance of the squared loadings of a factor (colums)on all variables (rows)in a factor matrix, which has the effect of differentiating the original variables from the extracted factor.

Factor analysis has been widely used in physical sciences such as geometry, ecology, hydrochemistry and molecular biology. Multivariate regreesion beteen two sets of PCs extracted from factor analysis is called redundancy analysis (Barthelomew, *et al.*, 2008).

4.3 Results

4.3.1 Assessing the Physical and Chemical Environmental Factors in *An. Gambiae* Breeding Sites

The physical environmental factors/conditions (pH, temperature, conductivity, transparency, total dissolved solids, and dissolved oxygen) of the mosquito breeding ecologies were measured on-site, using relevant portable field apparatus (See Chapter 2 for details) during the collection of the mosquito larvae. Furthermore, water samples were collected from the breeding sites and taken to the laboratory to assess the biological oxygen demand, as well as the levels of the chemical inorganic and organic contents (i.e. sulphates, phosphates, nitrites, nitrates, carbon content and oil and grease). In some of the sites visited, *An. gambiae* was found to be breeding alongside other forms of mosquito (mainly *Culex spp*). Therefore, total mosquito larvae and *An. gambiae* larval count (per litre of breeding water) were carried out to determine both the total mosquito larval and *An. gambiae* larval densities.

TABLE 4.1 Physico-chemical environmental factors in *An. gambiae* breeding sites located in study zone A (intensive agricultural areas)

Environmental Factors	Sampling Site			
	Site 1	**Site 2**	**Site 3**	**Combined Sites**
pH	7.17±0.06[a]	7.27±0.06	8.15±0.35	7.53±0.54
Temperature (°C)	35.90±0.02	34.80±0.01	36.33±0.09	35.68±0.79
Transparency (cm)	3.50±.0.00	2.00±0.00	2.10±0.00	2.53±0.84
Conductivity[b]	358.00±2.65	365.30±0.58	244.33±8.02	322.54±67.83
D O[c] (mg/L)	2.33±0.12	1.83±0.06	1.63±0.06	1.93±0.36
B O D[d] (mg/L)	1.73±0.12	1.23±0.06	1.13±0.06	1.36±0.32
T D S[e] (mg/L)	57.85±0.35	61.00±0.28	55.80±0.57	58.22±2.62
Sulphate (mg/L)	4.65±0.07	5.27±0.12	6.26±0.12	5.39±0.81
Phosphate (mg/L)	7.57±0.12	5.37±0.15	8.23±0.06	7.06±1.50
Nitrite (mg/L)	5.73±0.15	6.13±0.12	7.47±0.06	6.44±0.91
Nitrate (mg/L)	8.17±0.06	6.00±0.00	8.90±0.02	7.69±1.51
Carbon content (mg/L)	1.59±0.06	2.00±0.00	2.85±0.07	2.15±0.64
Oil & Grease (mg/L)	ND[f]	ND	ND	ND

[a] Data presented as mean ± SD; n=3

[b] Conductivity in µS/cm

[c] DO : Dissolved oxygen

[d] BOD: Biological oxygen demand

[e] TDS: Total dissolved solids

[f] ND: Not detected

TABLE 4.2 Physico-chemical environmental factors in *An. gambiae* breeding sites located in study zone B (domestic/residential areas)

Environmental Factors	Sampling Sites				
	Site 1	**Site 2**	**Site 3**	**Site 4**	**Combined sites**
pH	6.95±0.07[a]	7.35±0.07	7.30±0.14	7.15±0.13	7.19±0.18
Temperature (°C)	26.90±0.28	30.60±0.57	36.75±0.78	39.85±0.33	33.53±5.86
Transparency (cm)	1.90±0.00	1.20±0.00	6.50±0.00	1.30±0.00	2.73±2.54
Conductivity[b]	227.00±1.41	393.00±7.07	367.00±12.73	395.00±2.83	345.50±80.02
D O[c] (mg/L)	2.05±0.07	2.65±0.21	2.80±0.00	2.85±0.21	2.59±0.37
B O D[d] (mg/L)	1.05±0.07	1.20±0.14	1.50±0.00	1.75±0.07	1.38±0.31
T D S[e] (mg/L)	17.85±0.21	22.00±1.41	13.00±0.28	27.35±0.21	20.05±6.10
Sulphates (mg/L)	1.11±0.18	2.32±0.04	2.03±0.08	1.93±0.09	1.85±0.52
Phosphates (mg/L)	1.04±0.08	1.43±0.04	1.46±0.10	2.04±0.08	1.49±0.41
Nitrite (mg/L)	1.42±0.09	1.90±0.10	2.67±0.13	1.52±0.04	1.88±0.57
Nitrate (mg/L)	1.70±0.04	2.37±0.04	2.52±0.04	2.03±0.10	2.16±0.37
Carbon Content (mg/L)	1.05±0.06	2.03±0.08	0.99±0.13	0.80±0.12	1.22±0.55
Oil & Grease (mg/L)	ND[f]	ND	ND	ND	ND

[a.] Data presented as mean ± SD; n=3

[b] Conductivity in µS/cm

[c]DO : Dissolved oxygen

[d]BOD: Biological oxygen demand

[e]TDS: Total dissolved solids

[f]ND: Not detected

TABLE 4.3 Physico-chemical environmental factors in *An. gambiae* breeding sites located in study zone C (Petrochemicals/hydrocarbons laden areas).

Environmental Factors	Sampling Sites			
	Site 1	Site 2	Site 3	Combined Sites
pH	7.72±0.09[a]	7.96±0.08	7.63±0.09	7.77±0.17
Temperature (°C)	34.85±0.41	38.40±0.85	39.10±0.42	37.45±2.28
Transparency (cm)	2.10±0.00	2.50±0.00	2.70±0.00	2.43±0.31
Conductivity[b]	393.00±2.83	377.00±02.83	356.50±2.12	375.50±18.21
DO[c] (mg/L)	1.90±0.00	2.30±0.00	3.20±0.00	2.47±0.67
B O D[d] (mg/L)	0.90±0.00	0.85±0.00	0.90±0.00	0.87±0.06
T D S[e] (mg/L)	7.94±0.06	6.84±0.09	11.09±0.16	8.62±2.21
Sulphate (mg/L)	2.10±0.01	1.92±0.04	1.40±0.02	1.81±0.36
Phosphate (mg/L)	0.96±0.04	1.22±0.02	2.11±0.03	1.43±0.60
Nitrite (mg/L)	1.35±0.05	0.90±0.00	1.70±0.00	1.32±0.40
Nitrate (mg/L)	1.05±0.07	1.58±0.02	2.13±0.06	1.59±0.54
Carbon content (mg/L)	7.30±0.06	8.14±0.05	8.99±0.12	8.14±0.85
Oil & Grease (mg/L)	9.10±0.13	9.21±0.04	9.73±0.04	9.35±0.34

[a] Data presented as mean ± SD; n=3

[b] Conductivity in µS/cm

[c]DO : Dissolved oxygen

[d]BOD: Biological oxygen demand

[e]TDS: Total dissolved solids

[f]ND: Not detected

The results obtained (Tables 4.1, 4.2 & 4.3) showed little variation in the levels of the physical environmental factors (pH, temperature, conductivity, transparency and dissolved oxygen) across all the sampling sites in the three zones studied (A, B & C). However, the levels of Biological Oxygen Demand (BOD) in the sites located in study zone C (Table 4.3), which is petrochemical laden, were lower by 2 and 3-fold when compared to the levels recorded in sites within the studied zone A (agricultural) and B (residential) respectively (Table 4.1 & 4.2). The measured Total Dissolved solids (TDS) varied greatly across the three zones studied with sites in zone C (Table 4.3) having the lowest TDS concentration while sites in zone A have the highest. Generally, the TDS values recorded in zone C were more than 2 and 7-fold lower than those respectively observed in zone B and A. However within the zones, the concentration of TDS in each of the sites studied did not differ greatly. The concentration of sulphate, phosphates, nitrites and nitrates ions were highest in sites located in zone A and were 3.5-fold higher when compared to those recorded in both zone B and C. Their levels and variations in zone B were similar to those obtained in zone C. All the four inorganic ions appeared to exist in almost equal proportion in most of the sites across the zones. Next, the highest concentration of Carbon Content and Oil and Grease were observed in sites located in zone C. The carbon content in zone C was 3.7 and 6.6-fold greater than those observed in zones A and B respectively. Thus, the sites in the intensive agricultural zone have about 50% more carbon content than the sites in domestic/residential zone. There were no detectable levels of oil and grease at all in the sites within zones A and B. A graphical summary comparing these three zones is presented in Figure 4.1 below.

Table 4.4 *An. gambiae* larval densities within sites and across the three study zones

Sampling sites	Larval density (Per litre of Breeding water)	Study zone[a]
Site 1		
Site 2		A
Site 3		
Site 1		
Site 2		
Site 3		B
Site 4		
Site 1		
Site 2		C
Site 3		

[a]Zone: A, intensive agriculture; B, residential/domestic; and C, petrochemical breeding ecologies

Data in Table 4.4 showed that sites located in residential/domestic environments (zone B) have higher larval densities compared to the two other study zones. With the exception of site 2 of study zone C, *An gambiae* constitute the largest percentage of the total larval density in all the sites across the three study zones.

4.3.2 Relationship between Levels of Physico-Chemical Environmental Factors and Larval Density

In order to assess the degree of *An. gambiae* larval population (as a measure of breeding site productivity) and to evaluate the fitness of the larvae to further develop and emerge as pupae and adult *Anopheles* mosquito, the relationships and correlations between the levels of the physico-chemical environmental factors and larval density were examined within and across

the three different breeding ecologies or zones. Various statistical tools (See Chapter Two; Statistical Methods) in SPSS v.20 statistical package were employed to investigate these relationships and the outcomes of the various analyses are presented below:

4.3.2.1 Mean distribution of physico-chemical environmental factors and larval density

The result of one way ANOVA test shows that there is a high significant difference (p=0.000) in mean larval distribution of *An. gambiae* across the three study zones with zone B (residential/domestic) having the highest larval mean density of 75/L (Fig.4.1). The sampling sites within the intensive agricultural zone A were 2nd to those in zone B with a mean larval density of 29/L, which was about 38% of the larval productivity levels within zone B. The sites within the petrochemical laden breeding zone C had the lowest larval densities with a mean of about 1.9 and 5-fold lower than those observed for zones A and B respectively. The results of the Bonferoni Post-hoc test carried out to examine the pairwise mean distribution of larval density across the three study zones showed a significant differences (p=0.000) in mean larval distribution between zone A & B; B & C; and A & C.

The mean distribution of pH, temperature, conductivity DO, BOD, or transparency was not highly significant (Fig. 4.1) with p-values of 0.163, 0.492, 0.628, 0.234, 0.068 and 0.974 respectively across the three study zones.

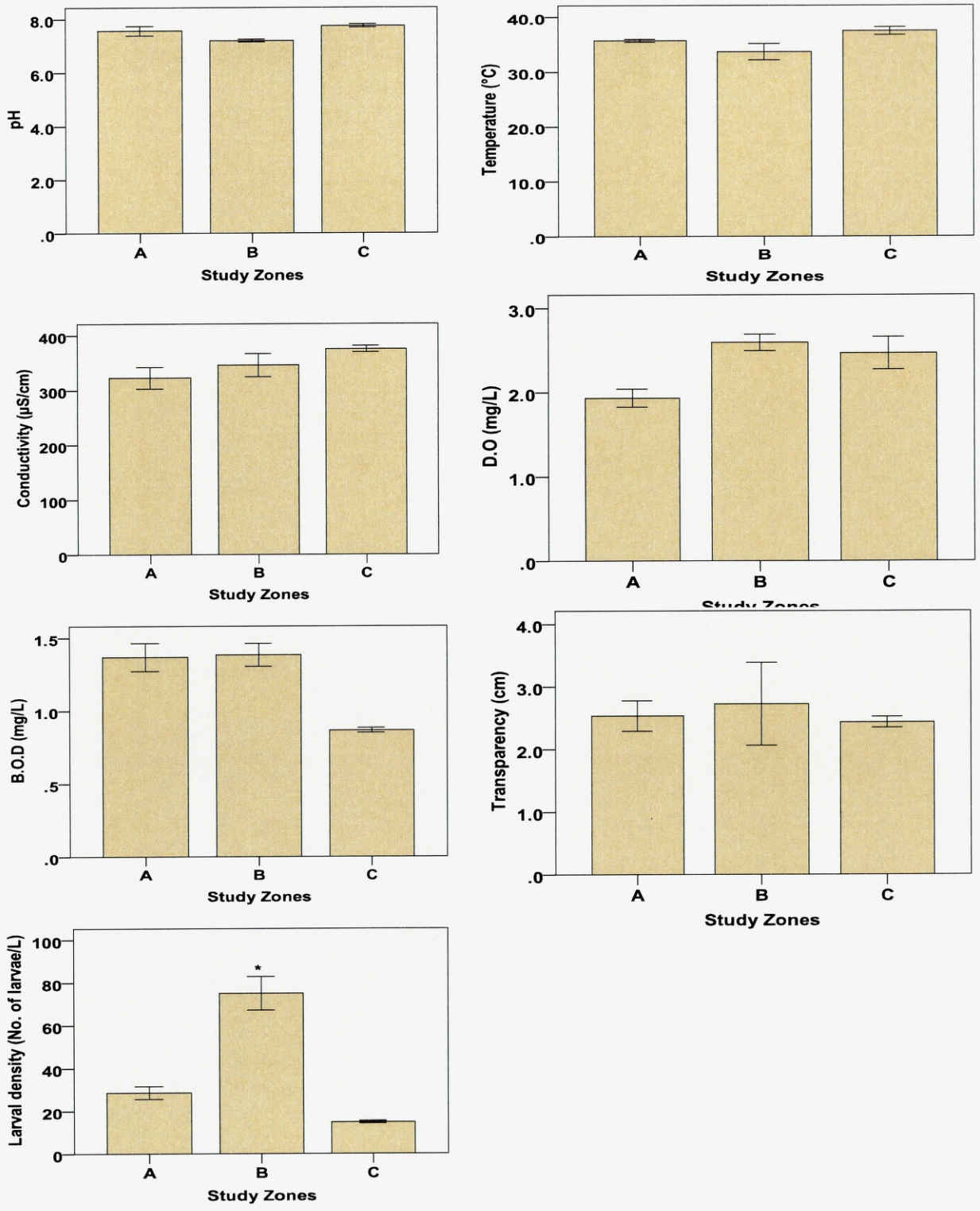

Figure 4.1 Distribution of mean larval density and environmental physical factors across three different *An. gambiae* breeding ecologies in Northern Nigeria. Zone A, intensive agriculture; zone B, domestic/residential; and zone C, petrochemical. * indicates that the value is significantly different ($p<0.05$) from those of the other zones.

Likewise, the differences in mean distribution of total dissolved solids, sulphates, phosphates, nitrites, nitrates, carbon content and oil and grease across the three study zones were highly significant (p=0.000) (Fig. 4.2). Also, the Bonferoni Post-hoc pairwise comparism tests showed that comparing mean distribution between zone A & B; A & C and B & C for most of the physical environmental factors were not highly significant. For instance, for pH, the zone-wise comparism between zone A against B, A against C and B against C were statistically not significant (p= 0.621, 1.000 and 0.218) respectively. For dissolved oxygen (DO), there was also no statistically significant differences (0.327, 0.620 and 1.000) in mean zone-wise comparisms between zone A against B, A against C and B against C respectively, while for BOD, A against B, A against C and B against C zone-wise comparisms recorded p-values of 1.000, 0.152 and 0.106 respectively. Lastly, same zone-wise comparisms for temperature, conductivity, and transparency were also not statistically significant (p=1.000). However, the zone-wise comparisms (A against B, A against C and B against C) for TDS and the environmental chemical factors (sulphates, phosphates, nitrites, nitrates, carbon content and oil and grease) were all statistically significant (p=0.000).

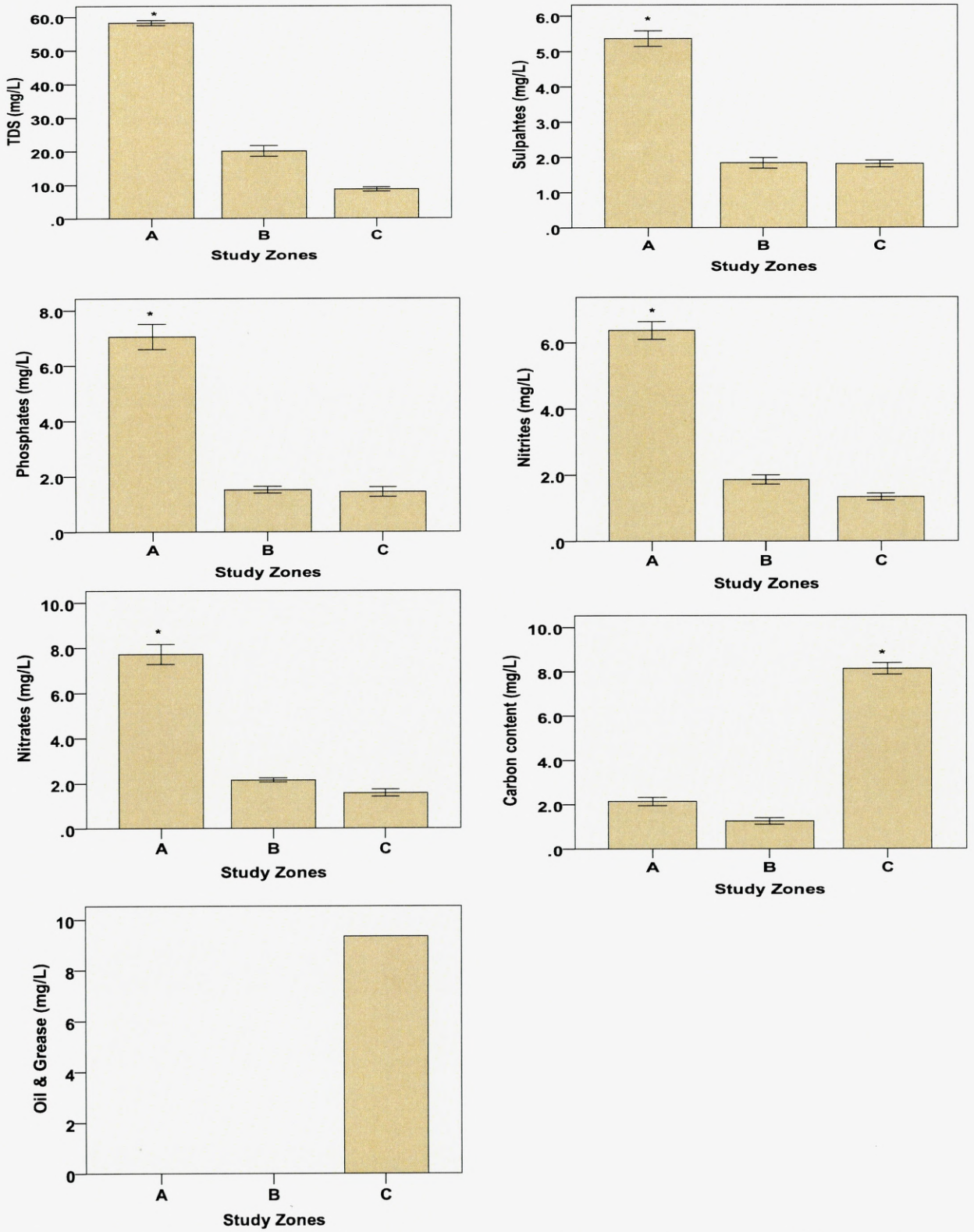

Figure 4.2 Distribution of chemical environmental factors across three different *An. gambiae* breeding ecologies in Northern Nigeria. Zone A, intensive agriculture; zone B, domestic/residential; and zone C, petrochemical. * indicates that the value is significantly different ($p<0.05$) from those of the other zones.

4.3.2.2 Association between each physico-chemical environmental factors and the larval density

The relationship between the individual physico- chemical environmental factors and larval density was conducted using Bivariate linear Regression analysis and data shown in Figures 4.3A and 4.3B illustrates the results obtained. The value of the coefficient of determination (R^2) describes the strength of the relationship between any particular environmental parameter and the larval density. The direction of the fitted lines in each plot describes the direction of the relationship. As shown in figure 2.3A, both pH and temperature were negatively correlated ($p<0.05$) with larval density, while transparency and BOD were positively associated ($p<0.05$) with larval density. The physical environmental parameters that appeared not to influence larval density were conductivity and dissolved oxygen.

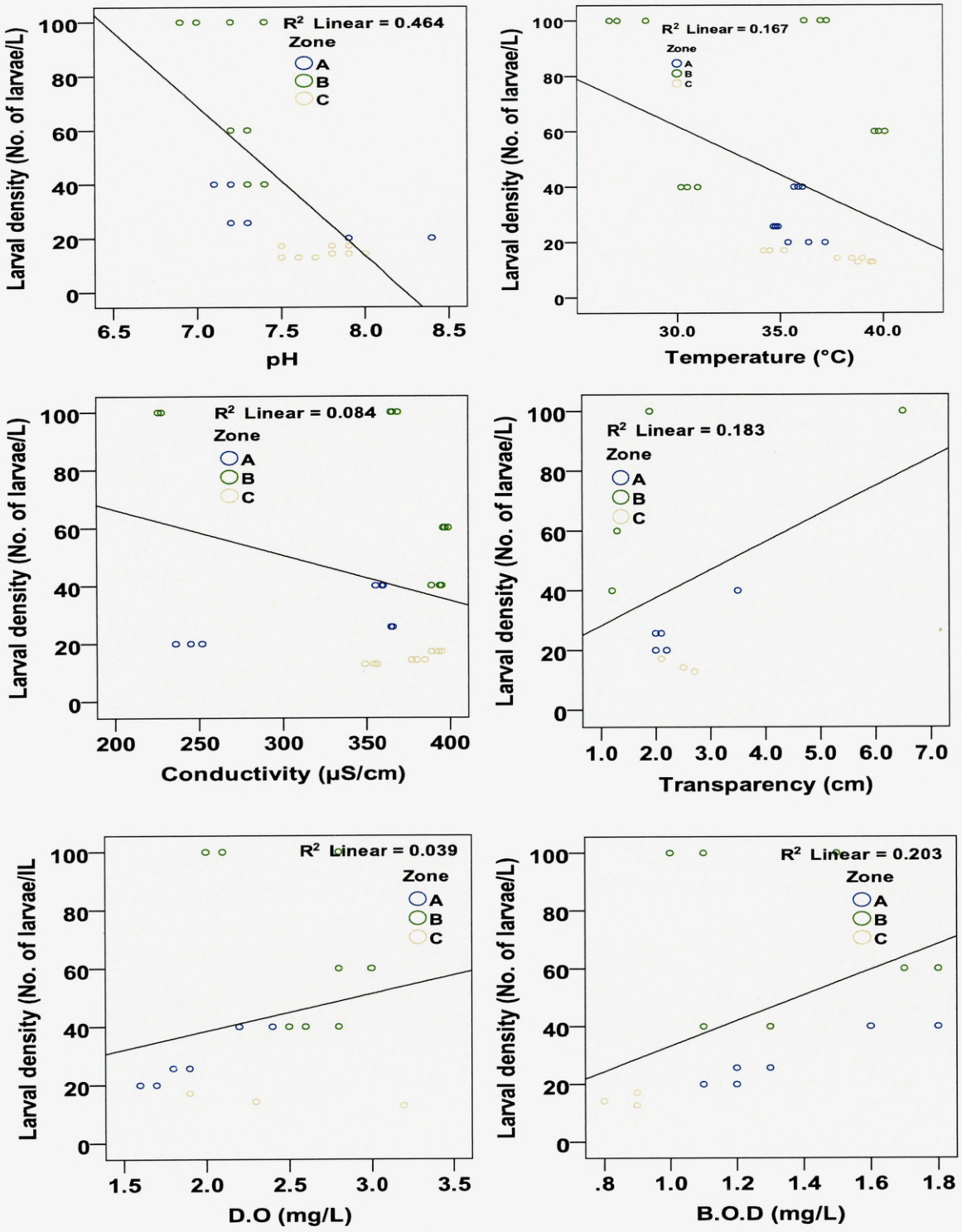

Figure 4.3A Correlations between *An. gambiae* larval density and physical environmental factors; pH, temperature, conductivity, DO BOD and transparency. The levels of these factors and the larval density were determined as described in Materials and Method section, in three different *An. gambiae* breeding ecologies in Northern Nigeria: Zone A, intensive agriculture; zone B, residential; and zone C, petrochemical.

Furthermore, the chemical environmental factors (sulphates, phosphates, nitrites, nitrates,) produced a moderate negative correlation ($p<0.05$) with *An. gambiae* larval density while carbon content and oil and grease produced highly statistical associations ($p<0.05$) with *An. gambiae* larval density. This suggests that as the levels of these chemical environmental parameters increases, the density of *An. gambiae* larvae decreases. As was observed with dissolved oxygen and conductivity, the total dissolved solids also produced no significant association with larval density (Fig.4.3B).

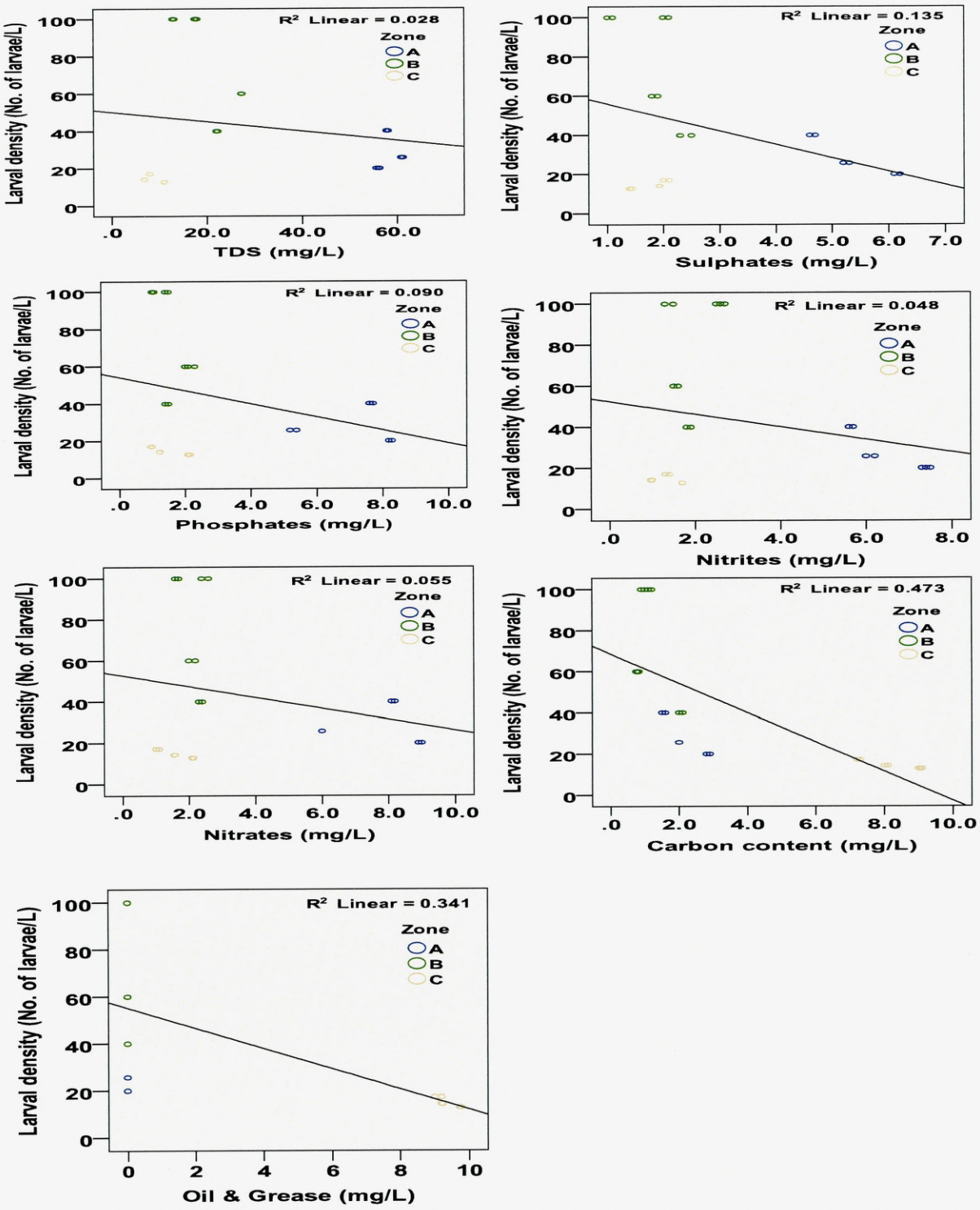

Figure 4.3B Correlations between *An. gambiae* larval density and chemical environmental factors (TDS, sulphates, phosphate nitrites, nitrates, carbon content and oil and grease). The levels of these factors and the larval density were determined, as described in Materials and methods section, from water samples collected three different *An. gambiae* breeding ecologies in Northern Nigeria: Zone A, intensive agriculture; zone B, residential; and zone C, petrochemical

4.3.2.3 Effect of the physico-chemical environmental factors on larval density

In order to deduce a model showing a combination of the different physico-chemical environmental factors that produce a combined singular effect on larval density, factor and regression in principal components analyses were carried out on the environmental factors and the larval density. Due to a strong colinearity in the physico-chemical variables, classical multiple regression analysis on these variables and the larval density failed to produce a reliable model estimate; the standard errors where inflated and thus the p-values associated with the contribution of the different covariates to the model were unreliable. Thus factor analysis was employed to extract principal components from the different model covariates (Physico-chemical variables) before running a regression on these components. The result of the factor analysis (Table 4.5) produced eight (8) principal components which explained 99% of the variability in the data (See Appendix for both Total Variance and Rotated Component Matrix) and thus only these components were retained as the model covariates for regression analysis.

Table 4.5 Extracted principal components and thier corresponding physico-chemical environmental factors

Principal components (PC)	Corresponding environmetal factors
PC1	TDS, Sulphates, Phosphates, Nitrites and Nitrates.
PC2	Carbon content nd Oil & grease
PC3	Conductivity
PC4	Temperature
PC5	Transparency
PC6	DO
PC7	pH
PC8	BOD

Thus component 1 represents contamination from pesticides and fertilizer application (Fertilizer and pesticides contaminants), 2 represents contamination from the sale, use, processing, and/or discharge of petrochemical/hydrocarbon products (petrochemical contamination) while principal components 3-8 represents the physical environmental variables.

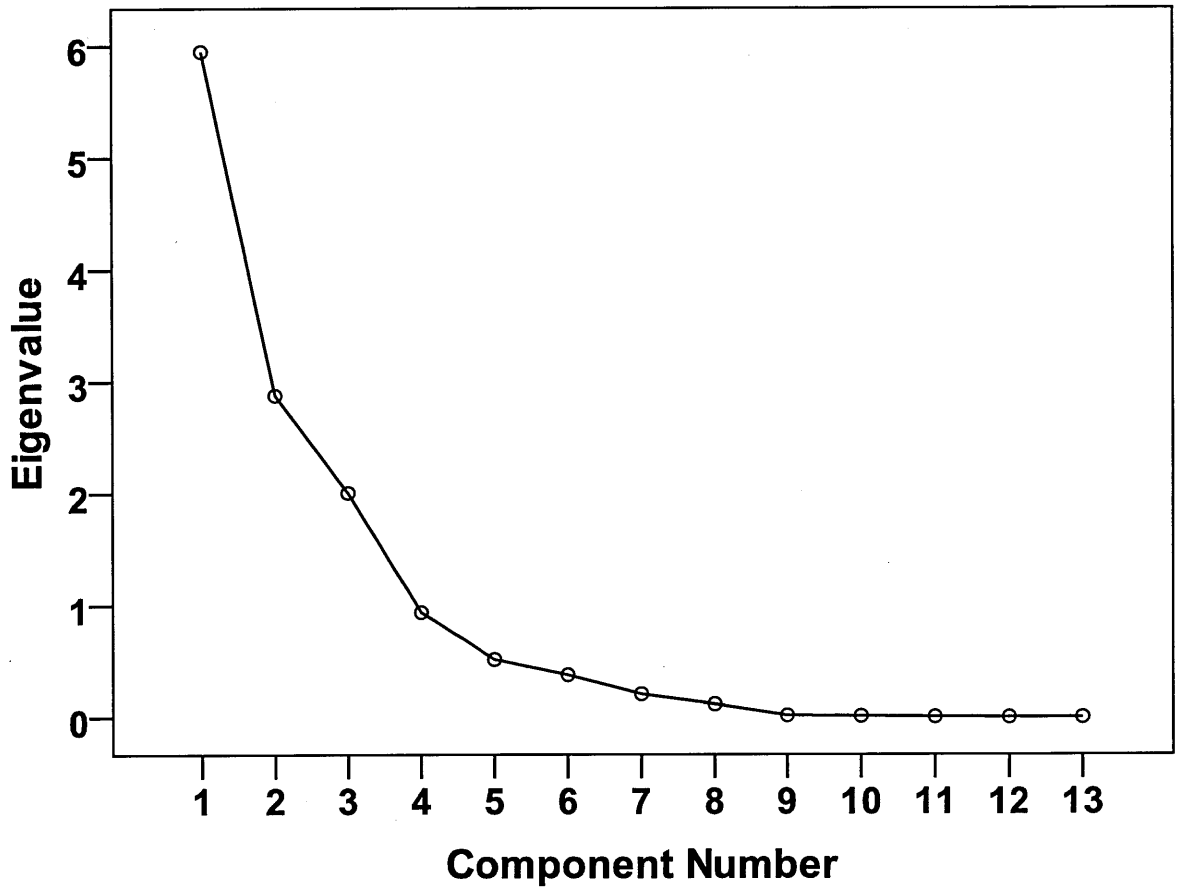

Fig. 4.4 Scree Plot of the extracted principal components from the factor analysis of the physico-chemical environmental variables. Components 1-8 explained 99% of the variability in the data.

A Scatter plot diagram (Fig. 4.5) of the scores of two of the factors showed that the data points clustered very well according to the three study zones were the data originated from.

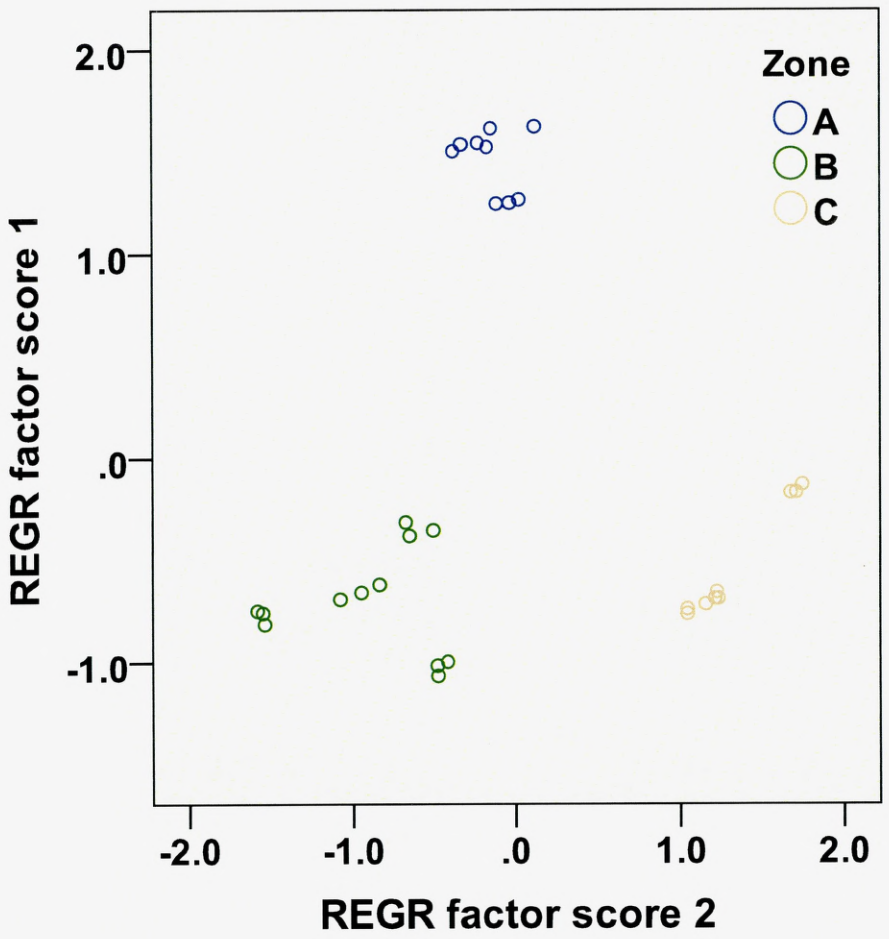

Fig. 4.5 Scatter plot of the scores of factor 1 and 2. Factor 1 represents pesticide and fertilizer contaminants while factor 2 represents petrochemical contaminants. Zone A; intensive agricultural areas, Zone B; Domestic/residential environments, Zone C; Petrochemical/hydrocarbon laden.

Finally, Regression in principal components was carried out between the extracted principal components from the physico-chemical environmental factors (PC 1-8; Table 4.5) and the larval density. The data in Table 4.6 showed the results obtained. From the results of the regression in principal components analysis (Table 4.6), only six out of the eight extracted principal components, i.e. those with the lowest significant values (p-values), produced a combined singular effect on larval density. Thus, pesticides and fertilizer contaminants (PC 1), petrochemical/hydrocarbon contaminants (PC 2) and the physical environmental variables; conductivity (PC 3), temperature (PC 4), transparency (PC 5), as well as pH (PC 7) produced the most significant combined effect on *An. gambiae* larval density in this part of Nigeria.

Table 4.6 Environmental Physico-chemical Factors or Components with Combined Effect on *Anopheles gambiae* Larval Density.

			95% Wald Confidence Interval		Hypothesis Test		
Parameter	**B**	**Std. Error**	**Lower**	**Upper**	**Wald Chi-square**	**df**	**Sig.**
Intercept	42.999	0.3749	42.264	42.739	13156.397	1	0.000
PC 1[a]	-13.200	0.3813	-13.948	-12.453	1198.567	1	0.000
PC 2	-22.414	0.3813	-23.162	-21.667	3455.792	1	0.000
PC 3	-10.962	0.3813	-11.709	-10.214	826.525	1	0.000
PC 4	-5.1520	0.3813	-5.899	-4.404	182.503	1	0.000
PC 5	12.301	0.3813	11.554	13.049	1040.873	1	0.000
PC 6	-0.136	0.3813	-0.883	0.612	0.126	1	0.722
PC 7	-7.684	0.3813	-8.431	-6.937	406.144	1	0.000
PC 8	0.051	0.3813	-0.696	0.799	0.18	1	0.893
Scale	4.216[b]	1.0886	2.542	6.993			

[a]: Refer to Table 4.5

[b] : Maximum likelihood estimate

4.3 DISCUSSION

This study demonstrated that the density of *An. gambiae* larvae was significantly influenced by some of the physico-chemical environmental factors that are associated with mosquito breeding ecologies. Densities of *An. gambiae* larvae varied significantly across the three study zones, with zone B (Table 3.1) recording the highest larval density. The mean levels of 7 physical environmental factors (pH, temperature, conductivity, transparency, DO, BOD, and TDS) and 6 chemical environmental factors (sulphates, phosphates, nitrites, nitrates carbon content and oil and grease) were determined in many breeding sites across the three study zones. While most organisms are known to respond differently to changes in the

physical conditions of their environments, the choice of the chemical environmental factors was made based on the type and nature of human related activities taking place in these zones. These activities were considered to be important in contributing to the presence of these chemical species in the mosquito breeding habitats. For instance, rain water runoffs from surrounding farmlands in the sampling and study zone A (intensive agricultural areas) could bring in high amount of nitrites, nitrates, sulphates and phosphates that are usually present in fertilizers and agro-pesticides, into the mosquito breeding site. Indeed, farmers interviewed nearby these breeding sites confirmed that fertilizers and various agro-allied pesticides (Chapter 3; Table 3.3) are constantly used in the area. All the farmers interviewed said they use both NPK (nitrate and phosphate-base) fertilizers together with organic manure from animal droppings, both of which usually contain nitrite, nitrate, and phosphate ions as their major chemical components. Furthermore, 80% of the respondents interviewed said they applied various pesticides such as Cypermethrin, Endosulfan, Fipronil, carbofuran, Dimethoate and Methyl-parathion. Chemical categorization analysis of these pesticides showed that they belonged to the four major classes of insecticides; carbamates, oganophosphates, organochlorines and pyrethroids and also contain carbon, sulphate and phosphate ions. Furthermore, runoffs from study zone C (petrochemical/hydrocarbon areas) is expected to contain high amount of carbon content and oil and grease. However, since some of these chemical species are also structural constituents of the many chemicals naturally present in the soil, appreciable levels of some of these chemical species were also detected in mosquito breeding sites located in study zone B (residential/domestic environments).

This study revealed that with the exception of oil and grease (which was detected only in zone C), all the breeding sites in the three studied zones contain various levels of these physical and chemical environmental factors. The levels of all the physical factors except

BOD (Fig. 4.1) were not significantly different across the three study zones. In contrast, the levels of all the chemical environmental parameters varied significantly across the three study zones (Fig. 4.2). The levels of TDS, sulphates, phosphates, nitrites and nitrates were significantly higher in sampling sites within study zone A. This could be explained by the use of nitrate and phosphates-base fertilizers as well as agro-allied pesticides in farmlands located around these mosquito breeding sites. The levels of carbon content and oil and grease were highest in breeding sites located in study zone C, which is consistent with the major types of human activities taking place. There is widespread sale, processing, use and discharge of petroleum products in this zone which appeared to be exacerbated by fuel (petrol or premium motor spirit) vending, a common practice in Northern Nigeria due to constant chronic fuel shortages. In addition, kerosene is a major domestic fuel in Nigeria and is normally sold by small retailers who are usually located within and/or around human habitats. Activities of automobile and motorcycle mechanics, who are often located around human habitation are also major additional sources of discharged spent fuel and lubricants into surrounding water bodies and mosquito breeding sites. All these contributed to the presence of very high levels of carbon content and oil and grease in breeding sites located in this zone relative to zones A and B.

The varying degree of *An. gambiae* larval densities from these breeding environments indicates that the physical and chemical environmental conditions of these breeding ecologies produced an effect on larval growth, fitness and survival. The breeding sites located zone B, which account for the highest transparency and DO and the lowest levels of most of the chemical environmental parameters (sulphates, phosphates, nitrates, nitrites and carbon content) observed, recorded the highest *An. gambiae* larval density. This was consistent with the findings of previous studies, which showed that *An. gambiae* prefers cleaner, clearer and uncontaminated breeding water (Sattler *et al.*, 2005). In this study, *An. gambiae* larval density

was negatively associated with pH and temperature and positively correlated with transparency and BOD. Majority of the sampling sites recorded temperatures of above 35°C. Thus, the result of this study agrees with previously reported observation that *An. gambiae* larval abundance increases with increasing temperature of up to 28-32°C above which it decreases (Depinay *et al.*, 2004; Munga *et al.*, 2005; Bayor and Lindsay, 2003). This means that temperatures of above 30°C affect the growth, fitness and survival of *An. gambiae* larvae. This observation is supported by the findings of Atkinson (1994) which showed that growth promoting enzyme-catalysed reactions decrease at high temperatures. The observed effect of pH on *An. gambiae* larval density in this study indirectly agrees with the findings of Ademoroti (2003) which reported that pH increases with increasing temperature due to concentration of carbon dioxide. This study revealed that *An. gambiae* was more abundant in clearer than in turbid water bodies. This result is similar to the findings of Mwangangi *et al.*, (2010) but contradicts the findings of McCrae (1984) which showed that *An. gambiae* females preferred turbid water to clear water for oviposition. Several factors such as insoluble particles of soil, organic matter, microorganisms, decaying vegetation and presence of other materials, are likely to contribute to the degree of transparency of *An. gambiae* breeding water. The levels of many of these materials are significantly lower in study zone B, as indicated by the concentration of TDS (Table 4.2) which explains the higher larval density recorded in this study zone. The level of BOD is dependent on the organic content of a water body. The observed effect of DO and BOD on larval density in this study is consistent with previous observations that higher aquatic organisms thrives better in water bodies with higher BOD than those with lower BOD levels. This is because higher levels of organic compounds lead to increase in microbial population which depletes dissolved oxygen (Goldman *et al.*, 1983).

The density of *An. gambiae* larvae was negatively associated with the levels of all the chemical environmental factors analysed in this study i.e. sulphates, phosphates, nitrites, nitrates, carbon content and oil and grease. This means that increase in the levels of these chemical species leads to decrease in the density of *An. gambiae* larvae. The fact that *An. gambiae* larval density decreases with increasing levels of these chemical species suggest that the mosquito larvae prefers breeding sites low in levels of these chemical species as evidenced by the high larval density recorded in study zone B. However, the presence of *An. gambiae* larvae in breeding sites containing high levels of these chemical species suggest a gradual potential emergence of tolerance of *An. gambiae* larvae to these environmental chemical factors. This is because Anopheles mosquitoes have not been previously associated with highly contaminated breeding ecologies. This may constitute a serious threat to the environmental management approaches to controlling malaria vector density. It was stated (section 4.1) above that environmental management strategy that aims to reduce adult vector population by targeting their aquatic immature (egg, larvae and pupae) is emerging as the mainstay of the contemporary approaches to malaria management especially in areas with high vectorial density. This strategy employed the use of various larvicidal chemical compounds to reduce larval density and by extension to minimize adult vector abundance (Gu and Novak, 2005). Therefore, potential tolerance to chemical factors arising from the use of agro-chemicals and petrochemical products reported in this study could pose a significant threat to the effectiveness of various larvicidal agents, especially those with similar structures and activity relationship with the environmental agro and petro-chemical products identified with the breeding sites ecology. In addition, adult mosquitoes emerging from these breeding ecologies could potentially be selected for both intrinsic and acquired resistance to agents used for their control, even in the absence of prior exposure or challenge.

Although all the results of the Linear Regression Analysis established the association of the individual physical and chemical environmental factors, the results of factor and followed by regression in principal components analysis complemented and augmented these observations by deducing a model of a combination of physico-chemical environmental factors that produced the most combined effect on *An. gambiae* larval density. According to this model (Table 4.6), both pesticide and fertilizer contaminants, petrochemical/hydrocarbon contaminants, together with some of the physical environmental factors produced the most singular combined effect on larval density. This suggests that fertilizer application for soil enrichment could be as important as the use of pesticides for agricultural pest control, in producing potential tolerance to these environmental factors by *An. gambiae* larvae. Many previous studies (Awolola *et al.*, 2005; Vulule *et al.*, 1994; Bruce-Chwatt, 1981; WHO, 1986; Hamon and Garett-Jones, 1963) have implicated various agricultural practices, including pesticides application, in the emergence and development of insecticides resistance in malaria vectors. The majority of these studies were restricted to adult mosquitoes and at the level of post-insecticide application. Therefore, there is a need for studies targeting the aquatic life stages at pre-insecticide exposure. Findings from such studies like this present one, would complement those of post-insecticides exposure by providing information on the various environmental factors that could serve as potential selection factors for the development and emergence of insecticides resistance.

Furthermore, several previous studies have investigated the relationship between various environmental, human and ecological indices and larval density, abundance as well as species variation and composition. These studies involved many mosquito species. For example, Mwangangi *et al.*, (2010) established a relationship between habitat characteristics and *Anopheles* larval abundance. Their findings showed that factors such as presence of other invertebrates, percentage *Azzolla* cover, distance to the nearest homestead, depth, and

turbidity produced a considerable effect on *Anopheles* larval density in Kenya. In a similar study, Kweka *et al.*, (2011) showed that seasonal variation in rainfall, algae cover, debris, chlorophyll-a, habitat depth and size all had significant impact on *Anopheles* species variability and habitat productivity. The study by Imbahale *et al.*, (2011) reported that *Anopheles* larvae were more likely to be abundant in man-made as oppose to natural habitats. Furthermore, rainfall intensity, salinity, presence of emergent plants, distance from the ocean and month of larval collection were all found to be significantly associated with the presence and abundance of *An. farauti* larvae in Solomon Islands (Bugoro *et al.*, 2011). Although, these studies were mostly restricted to the physical components of the environmental factors, their objectives nonetheless was to provide information for the practices and effects of environmental management approaches employed in malaria control. However, the use of chemical agents, larvicides for instance, to control aquatic life stages of mosquitoes is a major component of this environmental approaches. Therefore, studies such as this presented one, which investigated the impact of both physical and chemical environmental conditions of *An. gambiae* breeding ecologies on larval density and abundance, are necessary. This, as mentioned before, is because some of these environmental chemical factors could have similar structure and activity relationship with many of the chemical agents used in larval control. Thus, pre-exposure of *Anopheles* larvae to these environmental chemical parameters could be driving a potential development and emergence of tolerance to control agents of both larvae and adult mosquito. This could pose a significant challenge to the effectiveness of environmental management approaches to malaria vector control.

Finally, changes in the activities of detoxification enzymes (P450 monooxygenase, Glutathione-S-transferase and α and β-esterases) are major mechanism mosquitoes employ to produce tolerance to most insecticides. Studies have demonstrated higher activities of these enzymes in insecticides resistant relative to susceptible populations of *An. gambiae.* Higher

activities of detoxification enzymes are thought to result from either constitutive over-expression or up- regulation of detoxification genes, (Hemingway and Ranson, 2000; Chung *et al.*, 2011) as a result of induction following insecticide exposure. The environmental chemical factors involved in this study have been shown to have similar structures and activity relationship as well as similar routes of metabolism with almost all classes of insecticides use in mosquito control. Therefore, could differential levels of these chemical factors also produce changes in the levels of the detoxification enzymes activities in *An. gambiae*? In other words, could there be differences in detoxification enzymes activities in *An. gambiae* collected from breeding sites located in the three study zones A, B and C? And can the differences, if any, correlate with the levels of the environmental chemical factors? These questions can be answered by first carrying out detoxification enzyme assays on mosquito sampled from the three study zones and secondly, using appropriate statistical tools to investigate the relationships and correlations between the detoxification enzymes activities and levels of environmental chemical factors. The outcome of these kinds of analyses can be use to confirm or otherwise, the role of environmental physico-chemical conditions of *An. gambiae* breeding ecologies as potential selection factors for the emergence and development of insecticides resistance in *An. gambiae*.

4.5 Conclusion

Findings from this study suggests that *An. gambiae* breeding sites productivity (as measured by larval density) could be under strong influence from the physico-chemical environmental conditions of the mosquito breeding sites, and the levels and characteristics of these physico-chemical environmental factors are functions of the human related activities taking place within and around the mosquito breeding environments. The impact of these observations on the behaviour of the emerging mosquito, especially their response to the various insecticides-

based vector control programmes, could pose serious challenges to malaria management and control.

CHAPTER FIVE

5.0 Impact of Physico-chemical Environmental Conditions on the Activities of Detoxification Enzymes in *Anopheles Gambiae*

5.1 Induction of Detoxification Enzymes in Response to Environmental Xenobiotics

Environmental toxic substances generally referred to as xenobiotics poses a constant challenge to survival of organisms in their environments. Organisms are exposed to these substances through various means including; inhalation, ingestion, as well as physical contact. An elaborate three phase detoxification system is used by animals to defend themselves against the toxic effects of these substances. The three phase system metabolises the toxic substance into a less harmful one and excrete them out of the cell (Xu *et al.*, 2005). Among these detoxification phases, the phase I detoxification mechanism is the most elaborate; employing activities of enzymes belonging to the P450 family. In phase II, the by-product of phase I reaction are further detoxified by means of enzymes belonging to the GST and CE families (Misra *et al.*, 2011). The reactions involved in each phase increases the hydrophylicity of the xenobiotic compounds thereby facilitating their excretion. When organisms are exposed to environmental toxicants, a transcriptional response is activated which leads to upregulation of the genes involved in the detoxification machinery. This is called induction. Induction of detoxification enzymes in response to xenobiotic exposure has received greater attention in higher animals, because of its important implication in drug metabolism and discovery. In mammals, a number of transcription factors have been implicated in the response of detoxification enzymes to various environmental xenobiotics. Nuclear receptors, in particular the xenobiotic nuclear receptors, Pregnane X receptor (NR112), and constitutive androstone receptor (NR113) have been identified as the major regulators of all the enzymes involved in the three phases of xenobiotic metabolism. In

addition, the role of aryl hydrocarbon receptor (AHR) and aryl nuclear translocator (ARNT) in the regulation of detoxification genes has been established (Misra *et al.*, 2011). Studies on compounds that acts as ligands for AHR; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and other polycyclic aromatic hydrocarbons, provides much of the available information on the role of these transcription factors in the regulation of induction of detoxification genes (Schecter *et al.*, 2006). Other transcription factors mediating the induction of detoxification genes in many organisms include FXR, VDR and HNF4 nuclear receptors (Xu *et al.*, 2005; Pascussie *et al.*, 2008), nuclear erythroid-related factor2 (NrF2) and CNC-bz1p transcription factors (Nguyen *et al.*, 2009; Sykiotis and Bohmann 2010).Most studies on the mechanism of induction of detoxification enzymes in higher animals used humans and mice as models.

Studies on induction of detoxification enzymes in insects have tended to focused more on adaptation; how a particular strain of insect has adapted to a particular environment which could then selects it for insecticides resistance (Perry *et al.*, 2011). However, evidences have emerged that insects like other higher animals have the ability to regulate the transcription of detoxification genes in response to environmental xenobiotics. Studies have been conducted and many more are still ongoing to describe the peculiar nature of this regulation in insects (Misra *et al.*, 2011).

Studies have indicated that though organisms have the ability to synthesize several forms of P450 isoenzymes during their lifetime, it is unlikely that all these are present at any one given time. Available evidences now suggest that some of the P450s are used to maintain the normal physiological activities of the organisms while others are produced only if needed for specific functions. For the purpose of convenience, Nobert and Colleagues (1981) classified these two forms of P450 system into native and defensive forms. Because of substrate overlap, the native forms could also metabolise xenobiotics at early stages of exposure but this function is more effectively carried out by the defensive forms. Because of his

observation, it is now believed that the defensive forms of P450s are produced as a result of induction in response to xenobiotic exposure. Data on the mechanisms of enzyme induction suggest that the enzymes produced as a result of induction are not used exclusively for the metabolism of the inducing substances but have more general and wider targets. For instance, in mice, it was found that about 20 forms of drug metabolising P450 were produced in response to induction of liver aromatic hydrocarbon hydroxylase by 3-methyl chloranthene (David *et al.*, 2013). This would infer that induction of a particular detoxification enzyme by a specific compound can lead to activation of several related enzymes designed to metabolise a variety of compounds related or otherwise, rather than the specific agent that triggered the induction in the first place. The phenomenon of induction is not limited to the P450 monooxygenases. Induction is a widespread response involving varieties of enzymes. Other enzymes which are also being investigated include GST, epoxide hydratase, uridine 5-diphosphate glucuronyl (UDPGA) transferases, and CEs (Hemingway *et al.*, 2004).

The first documented evidence of enzyme induction in insects was given by Agnosin and Dinamarca (1963), in which they reported an increased activity of NAD Kinase in *Triatoma infestans* after exposure to DDT. Evidences are beginning to emerge of the induction of the three major detoxification enzyme systems in insects; P450 cytochromes, GST and CEs. A comprehensive review on the incidences of induction of these enzymes by various xenobiotics in many species of insects have well documented (David *et al.*, 2013). *Aedes* mosquitoes and *Drosophila* have featured most prominently in many recent studies involving induction of one or more of the detoxification enzymes in response to various environmental xenobiotics. Benzothiazol and Pentachlorophenol were found to increase the tolerance of *Aedes albopictus* larvae to different types of synthetic insecticides including cabaryl, rotenone and temephos (Suwanchaichinda and Brattsten, 2001; 2002). The tolerance was found to correlate with increase in the activity of P450. The tolerance of *Aedes aegypti* larvae

to organophosphate insecticide after a prior exposure to artrazine was reported by Boyer *et al.*, (2006). Poupardin and Colleagues (2008) also reported a moderate to high tolerance of *Aedes aegypti* to organophosphate and pyrethroid insecticides following exposure to Fluoranthene and the heavy metal, Copper. In a similar study, the herbicide glyphosate and the aromatic hydrocarbon benzo[a] pyrene, were found to significantly increase the activities of detoxification enzymes and cause the induction of detoxification genes in *Aedes aegypti* larvae expose to the insecticides imidacloprid, permethrin and propoxur (Riaz *et al.,* 2009). Induction of various detoxification enzymes was demonstrated in *Drosophila* exposed to chemicals such as barbiturates and Phenobarbital in studies using microarray-based approaches (Le Goff *et al.*, 2006). In most of these studies, a correlation between induction and increase in the activities of the induced enzymes was established. In some cases, DNA microarray was used to investigate the specific detoxification genes induced by particular xenobiotics (Poupardin *et al.*, 2008).

However, *An. gambiae*, a major malaria vector, has not featured prominently in studies involving the relationships between xenobiotic exposure and induction of detoxification enzymes. Although, the inductive ability of detoxification genes in *An. gambiae* in response to insecticides like permethrin was demonstrated by Vontas *et al.,* (2005), the role of prior exposure to varieties of environmental chemicals has not been largely investigated. These kinds of studies are especially important given the ability of *Anopheles* mosquitoes to thrive in varieties of contaminated environments. Therefore, this study examine the response of An. gambiae detoxification enzymes to levels of vrious physico-chemical environmental factors present in their breeding ecologies.

5.2 Results

5.2.1 Assessment of Activity Profiles of Detoxification Enzymes across the three Life Stages of *An. gambiae*

Table 5.1. The activities of Cytochrome P450 determined from the three life stages of *An. gambiae* collected from breeding sites across three different study zones.

Sampling sites	Life Stages of *An. gambiae*			Study zones[a]
	Larvae	Pupae	Adult	
Site 1	0.097±0.0050[b]	0.115±0.0060	0.084±0.0050	
Site 2	0.083±0.0060	0.133±0.0050	0.064±0.0030	A
Site 3	0.125±0.0050	0.149±0.0030	0.099±0.0020	
Site 1	0.027±0.0029	0.041±0.0021	0.018±0.0031	
Site 2	0.042±0.0026	0.053±0.0021	0.041±0.0029	B
Site 3	0.017±0.0015	0.021±0.0006	0.013±0.0020	
Site 4	0.036±0.0000	0.043±0.0012	0.031±0.0021	
Site 1	0.143±0.0035	0.157±0.0021	0.172±0.0023	
Site 2	0.162±0.0025	0.181±0.0023	0.201±0.0036	C
Site 3	0.198±0.0006	0.236±0.0040	0.293±0.0035	

[a]Zone: A, intensive agriculture; B, residential/domestic; and C, petrochemical breeding ecologies

[b] Data presented as mean ± S.D (EUP450/mg protein) for three determinations

Table 5.2 The activities GST determined from the three life stages of *An. gambiae* collected from breeding sites across three different study zones.

Sampling sites	Life Stages of *An. gambiae*			Study zones[a]
	Larvae	Pupae	Adult	
Site 1	0.212±0.0040[b]	0.247±0.0040	0.182±0.0030	A
Site 2	0.298±0.0040	0.346±0.0050	0.243±0.0030	
Site 3	0.566±0.0040	0.714±0.0140	0.487±0.0040	
Site 1	0.0097±0.0006	0.0133±0.0015	0 .0050± 0.000	B
Site 2	0.0193±0.0012	0.0240±0.0030	0.0173±0.0023	
Site 3	0.0041± 0.0002	0.0056±0.0002	0.0036±0.0001	
Site 4	0.011±0.0012	0.015±0.0021	0.012±0.0010	
Site 1	0.018±0.0030	0.024±0.001	0.016±0.000	C
Site 2	0.015±0.0000	0.019±0.001	0.011±0.001	
Site 3	0.048±0.0030	0.058±0.002	0.041±0.001	

[a]Zone: A, intensive agriculture; B, residential/domestic; and C, petrochemical breeding ecologies

[b]Data presented as mean ± S.D (μmol/min/mg protein) for three determinations

Table 5.3 The activities of α- Esterase determined from the three life stages of *An. gambiae* collected from breeding sites across three different study zones

Sampling sites	Life Stages of *An. gambiae*			Study zones[a]
	Larvae	Pupae	Adult	
Site 1	0.0081±0.00012[b]	0.0094±0.00006	0.0081±0.00015	
Site 2	0.0096±0.00040	0.0104±0.0003	0.0083±0.0003	A
Site 3	0.0096±0.00040	0.016±0.0030	0.0076±0.0004	
Site 1	$0.00083\pm3.25\times10^{-5}$	$0.00095\pm1.73\times10^{-5}$	$0.00080\pm1.73\times10^{-5}$	
Site 2	$0.00092\pm1.15\times10^{-5}$	$0.0016\pm0.25\times10^{-5}$	$0.00089\pm2.08\times10^{-5}$	
Site 3	0.00027±0.00000	$0.00039\pm58\times10^{-5}$	$0.00022\pm2.08\times10^{-5}$	B
Site 4	$0.00071\pm2.1\times10^{-5}$	$0.00084\pm1.53\times10^{-5}$	$0.00062\pm1.0\times10^{-5}$	
Site 1	$0.00046\pm2.65\times10^{-5}$	$0.00056\pm2.31\times10^{-5}$	$0.00039\pm58\times10^{-5}$	
Site 2	$0.00030\pm3.21\times10^{-5}$	0.00035±0.00000	$0.00024\pm2.52\times10^{-5}$	C
Site 3	$0.00074\pm4.16\times10^{-5}$	$0.0010\pm2.3\times10^{-5}$	0.00084±0.00000	

[a]Zone: A, intensive agriculture; B, residential/domestic; and C, petrochemical breeding ecologies

[b] Data presented as mean ± S.D (μmol 1-Naphthol/min/mg protein) for three determinations

Table 5.4. The activities of β- Esterase determined from the three life stages of *An. gambiae* collected from breeding sites across three different study zones.

Sampling sites	Life Stages of *An. gambiae*			Study zones[a]
	Larvae	Pupae	Adult	
Site 1	0.0040±0.00027[b]	0.0024±0.00031	0.0038±0.00035	A
Site 2	0.0068±0.00060	0.0034±0.00042	0.0066±0.00050	
Site 3	0.0060±0.00029	0.0035±0.00021	0.0052±0.00031	
Site 1	$0.00037\pm1.53\times10^{-5}$	$0.00026\pm1.55\times10^{-5}$	0.00035±0.00000	B
Site 2	$0.00052\pm2.52\times10^{-5}$	$0.00037\pm1.53\times10^{-5}$	$0.00051\pm2.89\times10^{-5}$	
Site 3	$0.00015\pm1.53\times10^{-5}$	0.00009±0.00000	$0.00012\pm1.54\times10^{-5}$	
Site 4	$0.00033\pm1.15\times10^{-5}$	$0.00016\pm2.3\times10^{-5}$	0.00030±0.00001	
Site 1	$0.000087\pm252\times10^{-5}$	$0.000064\pm580\times10^{-5}$	$0.000086\pm580\times10^{-5}$	C
Site 2	$0.000062\pm252\times10^{-5}$	0.000037±0.000000	$0.000056\pm461\times10^{-5}$	
Site 3	0.00018±0.00001	$0.00011\pm57\times10^{-5}$	$0.00022\pm3.06\times10^{-5}$	

[a] Zone: A, intensive agriculture; B, residential/domestic; and C, petrochemical breeding ecologies

[b] Data presented as mean ± S.D (μmol 2-Naphthol/min/mg protein) for three determinations

The results presented (tables 5.1-5.4) showed that the activities of the four detoxification enzymes vary not only across the three study zones but also across the three life stages studied. Across the life stages, P450 activities appears to be higher in the pupal stages of *An. gambiae* from breeding sites located in study zone A and B, while sites located in study zone C, the adult stage has the highest P450 activities. The P450 activities of the pupal stage of *An. gambiae* from breeding sites located in study zones A and B were about 80% higher than that

of the adult stages from the same zones. In contrast however, the P450 activity of the adult stage of *An. gambiae* from sites located in study zone C was approximately 2-fold higher when compared to the activity at the larval and pupal stages of the same zone. Furthermore, the distribution of P450 activities across the three study zones varies, with sites located in zone C (petrochemical laden) having the highest activities. The P450 activities of *An. gambiae* from breeding sites located in study zones A and B were about 2 and 4-fold lower respectively, when compared to those recorded in study zone C (Table 5.1). The distribution of the activities of GST and α and β-esterases across the three study zones showed a marked contrast when compared to that of P450. Study zone A account for the highest activities of the three enzymes compared to zones B and C. Indeed, activities of these three enzymes in the three life stages of *An. gambiae* from sites located in zone A were approximately 9-fold higher than those of zone B and C (Table 5.2-5.4). Finally, with the exception of β-esterase, the distribution of the activities of GST and α-esterase across the life stages is similar to that observed in P450; the activities were higher in the pupal stage compared to the other two life stages. Refer to Figures 5.1, 5.3, 5.5 & 5.7 for graphical comparism of the distribution of these enzymes across the three study areas.

5.2.2 Relationship between Levels of Physico-chemical Environmental Factors and the Activities of Detoxification Enzymes in *An. gambiae*

In order to evaluate the role of physico-chemical environmental conditions of *An. gambiae* breeding ecologies as potential selection factor for the emergence and development of insecticides resistance, the effects of levels of physico-chemical environmental factors on the activities of detoxification enzymes was examined in the three life stages of *An. gambiae* sampled from three different breeding ecologies in northern Nigeria (Table 3.1). Various statistical tools (See Chapter Two; Statistical Methods) in SPSS v.20 were employed for the analyses and the results are presented below:

5.2.2.1 Mean Distribution of the Detoxification Enzymes and Association between the Enzymes and the Environmental Factors

a) P450 Monooxygenase Enzyme

i) Larval Stage

In order to investigate the differences in mean distribution of P450 monooxygenase activity at the larval stage of *An. gambiae* across the three studied zones, one way ANOVA test was used. As shown in Fig 5.1, the differences was indeed significant (p=0.000) with study zone C (petrochemical laden) having the highest mean larval activity. The mean P450 activities of zone A and B were 1.7 and 4.3-fold lower than that of zone C.

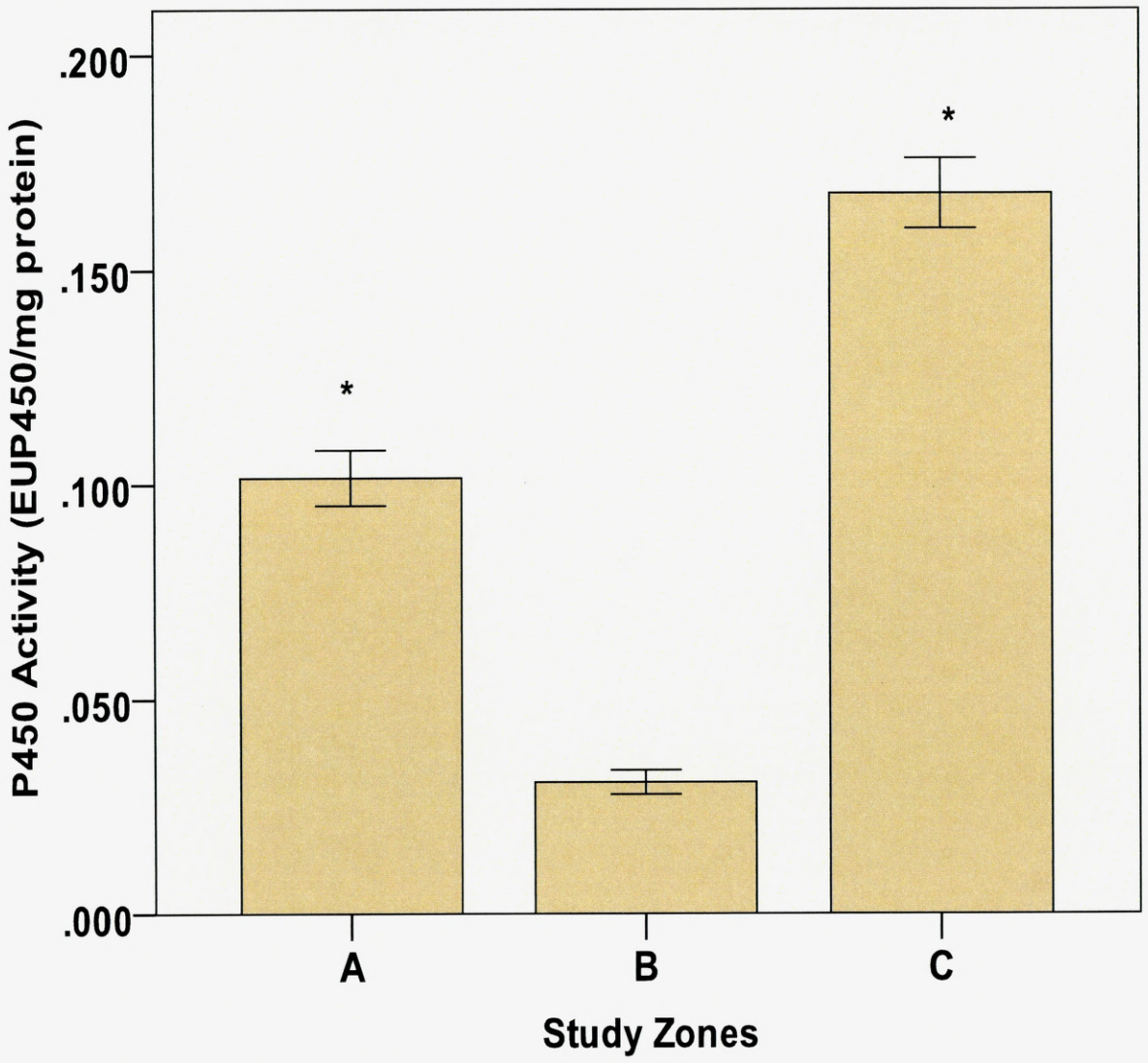

Fig. 5.1 Comparative mean distribution of P450 monooxygenase activity at the larval stage of *An. gambiae* from three different breeding ecologies in Northern Nigeria: Zone A, intensive agriculture; zone B, domestic/residential; and zone C, petrochemical. The P450 activity was measured using monooxygenase titration assay with methanol solution of $3,3^{1}5,5^{1}$ –tetramethyl benzidine in 0.25M acetate buffer (pH 5.0) as substrate. * indicates that the value is significantly different ($p<0.05$) from that of the other zone.

Additionally, Bonferoni Post-hoc pair-wise comparism test showed highly significant differences in mean larval P450 activities between zone A and B (P=0.008) and B & C (P=0.000). The difference between A and C was moderately significant (P=0.160).

The correlations between each physical and chemical environmental factors and larval P450 activity were conducted using Bivariate Linear Regression analysis and data shown in figures

5.2A and 5.2B illustrates the results obtained. The p-values as well as the coefficient of determination (R^2) describes the strength of the association or correlation between any particular environmental parameter and larval P450 activity. The direction of the fitted lines in each dot plot describes the direction of this association. pH and temperature were statistically positively associated (p= 0.000 and 0.010) respectively with larval P450 activities while BOD showed significant (p=0.000) negative correlation (Fig. 5.2A). There were no significant associations (p= 0.655, 0.806 and 0.416) between Conductivity, DO and transparency respectively, and larval P450 activity ($p<0.05$).

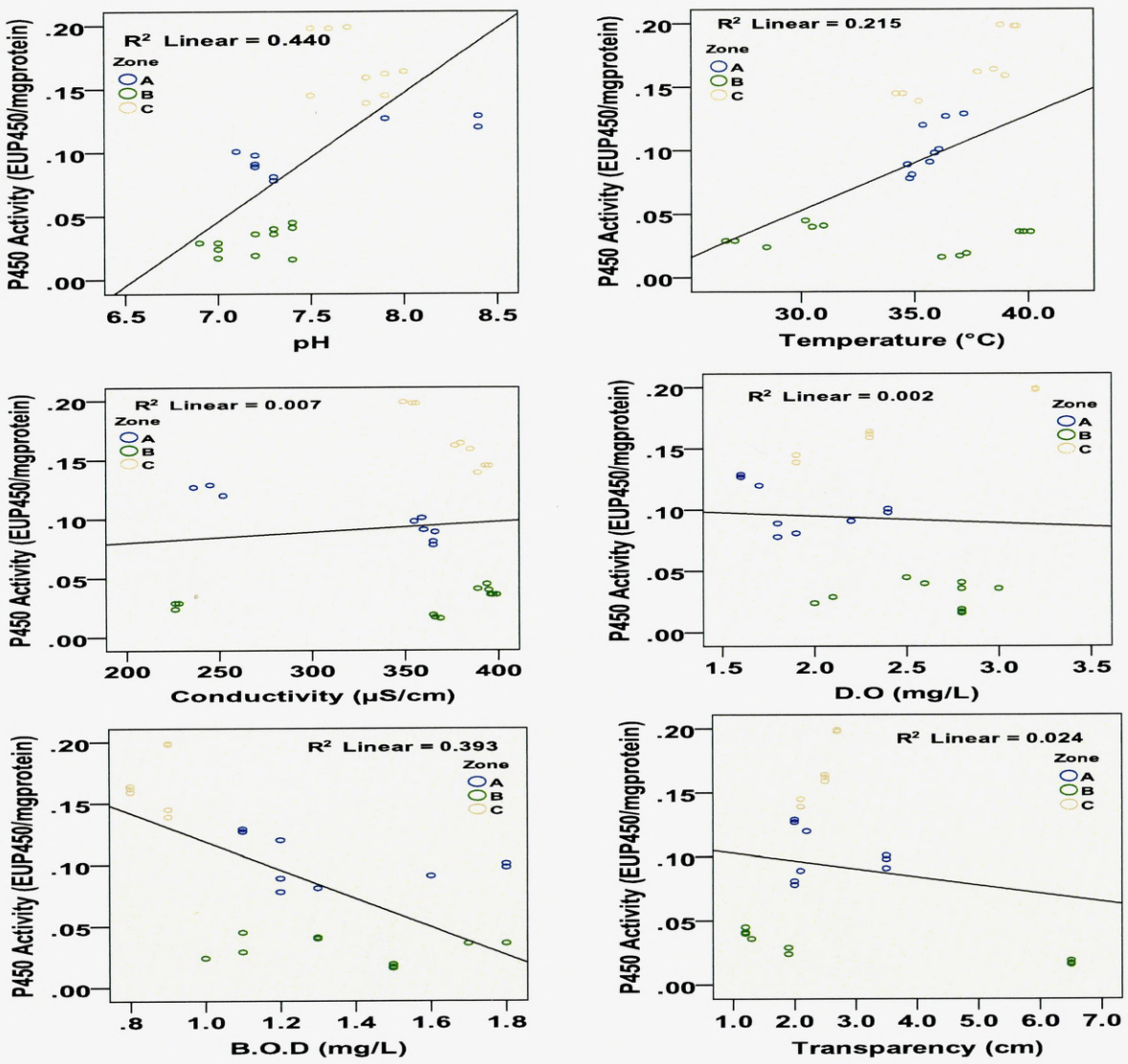

Fig. 5.2A Correlations between larval P450 activity and physical environmental factors; pH, temperature, conductivity, DO, BOD and transparency. The levels of these factors and P450 activity were determined, as described in Material and Methods section, in three different *An. gambiae* breeding ecologies in Northern Nigeria: Zone A, intensive agriculture; zone B, residential and zone C, petrochemical.

Furthermore, the chemical environmental factors; TDS, sulphates, phosphates, nitrites, and nitrates were not significantly associated (p= 0.540, 0.616, 0.465, 0.891 and 0.743) respectively, with larval P450 activity while carbon content and oil and grease were significantly positively correlated (p=0.000) with larval P450 activity. This means increase in the levels of carbon content and oil and grease produced increased larval P450 activity (Fig. 5.2B).

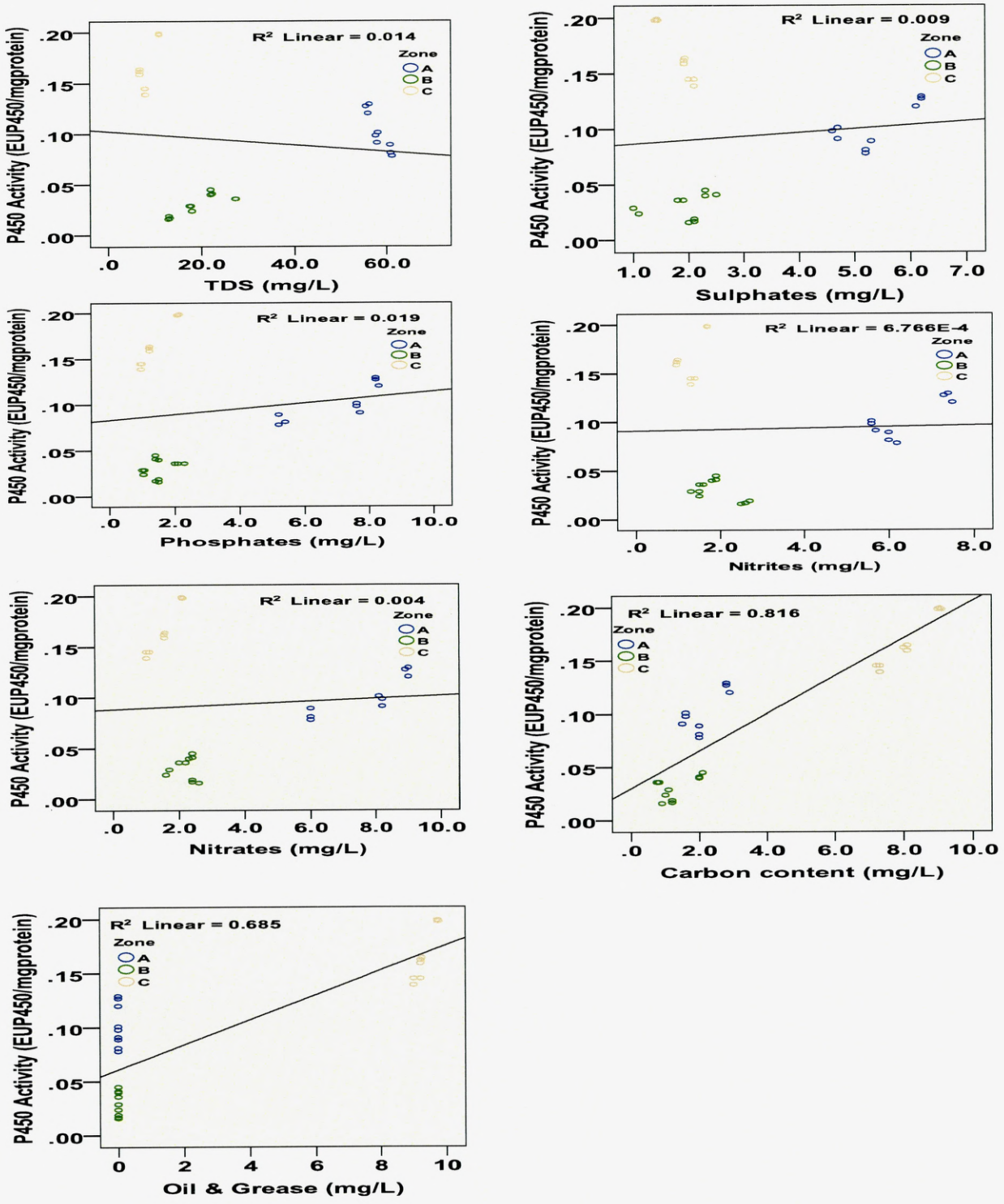

Fig. 5.2B Correlations between larval P450 activity and chemical environmental factors; TDS, sulphates, phosphates, nitrites, nitrates, carbon content and oil and grease. The levels of these factors and P450 activity were determined, as described in Material and Methods section, in three different *An. gambiae* breeding ecologies in Northern Nigeria: Zone A, intensive agriculture; zone B, residential and zone C, petrochemical.

ii) Pupal Stage

The same sets of analyses carried out to investigate the relationship between levels of physico-chemical environmental factors and P450 activities at the larval stage, were also conducted at the pupal stage of *An. gambaie*, and the results obtained were very similar to those recorded for the larval stage. Firstly, the distribution of mean P450 activity at this stage was similar to that of larval stage, with significant differences (p=0.000) in mean activity distribution across the three study zones. As in the larval stage, the breeding sites located in study zone C (petrochemical laden) also recorded the highest pupal P450 activity. Additionally, the Bonferoni Post-hoc pairwise comparism test showed highly significant differences between zone A & B and B & C (p=0.006 and 0.000) respectively, while the difference between A & C was not highly significant (p=0.730). Furthermore, the results of the Linear Regression analysis, carried out to examine the correlations between each environmental parameter and pupal P450 activity, were similar to those recorded in the larval stage.

iii) Adult stage

The same types of analyses were carried out on P450 activity at the adult stage of *An. gambiae* as in the larval and pupal stages. The results and observations made were similar to those of the two previous life stages. Same pattern of distribution of P450 activity was recorded across the three study zones and similar patterns of correlations or association were recorded for the individual environmental factors against adult stage P450 activity.

b). Glutathione S-Transferase (GST) Enzyme

i) Larval Stage

The result of one way ANOVA test carried out to examine the distribution of mean GST activity at the larval stage of *Anopheles gambiae* showed that there was highly significant differences (p=0.000) in mean GST activity distribution across the three study zones with breeding sites located in zone A having the highest mean activity (Fig. 5.3).

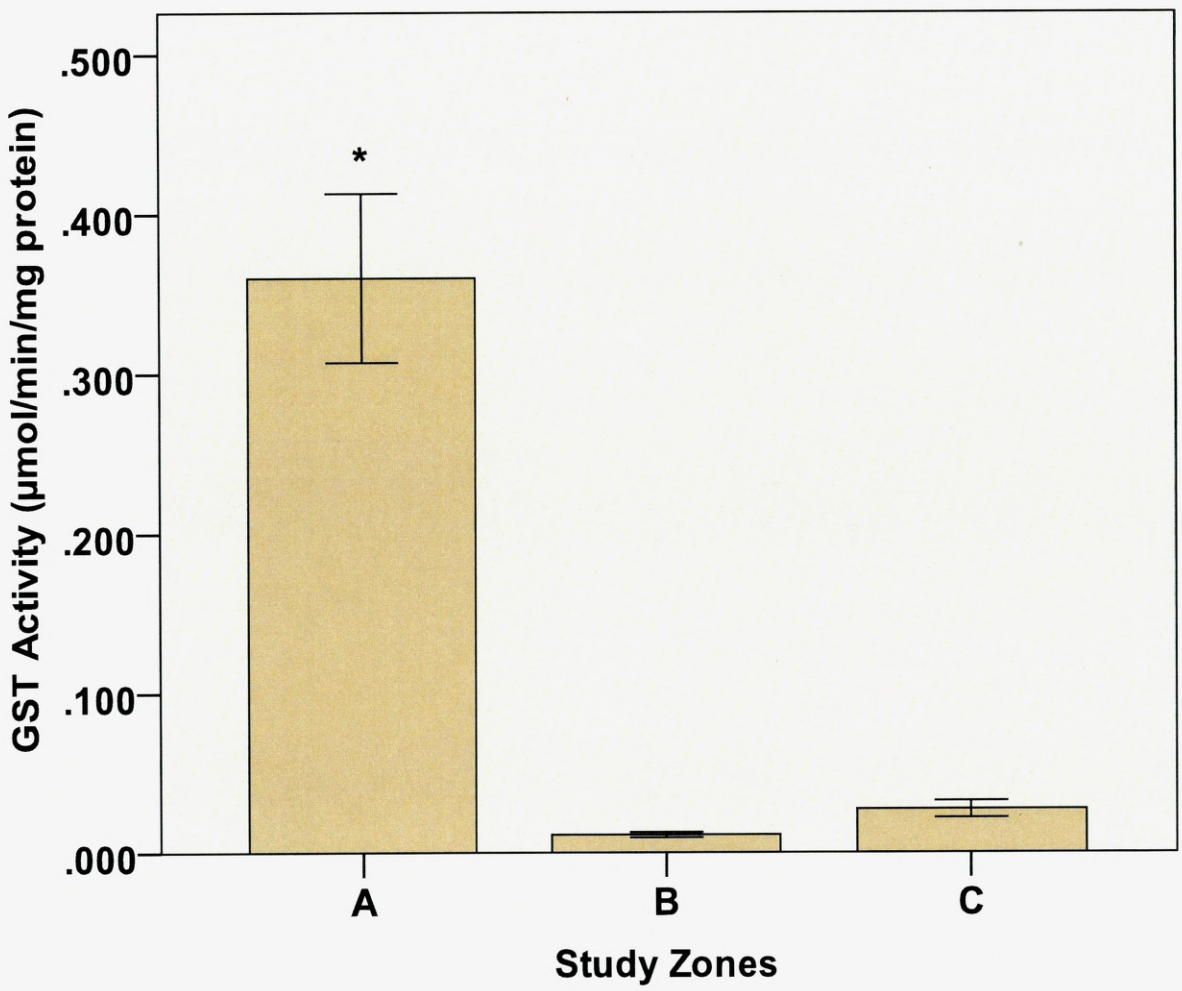

Fig. 5.3 Comparative mean distribution of GST activity at the larval stage of *Anopheles gambiae* from three different breeding ecologies in Northern Nigeria: Zone A, intensive agriculture; zone B, domestic/residential; and zone C, petrochemical. The GST activity was measured using CDNB assay with 63mM methanol solution of chlorodinitrobenzene as substrate. This substrate concentration was mixed with 10mM GSH in 0.1M phosphate buffer to produce the working solution for the assay. * indicates that the value is significantly different ($p<0.05$) from those of the other zones.

The difference in mean GST activity distribution between *An. gambiae* larvae from breeding sites located in study zone A & B and A & C were highly significant (p= 0.010 and 0.021) respectively, while the difference between zone B & C was not (p=1.000) (Fig. 5.3).

Furthermore, the results of the Bivariate Linear Regression analysis, which was use to investigate the association between each physico-chemical environmental factors and larval GST activity showed that the physical environmental factors; pH, conductivity, and DO were all significantly associated (0.011, 0.008 and 0.000) respectively, with larval GST activity, while temperature, BOD and transparency (p= 0.654, 0.713 and 0.551) respectively were not (Fig. 5.4A)

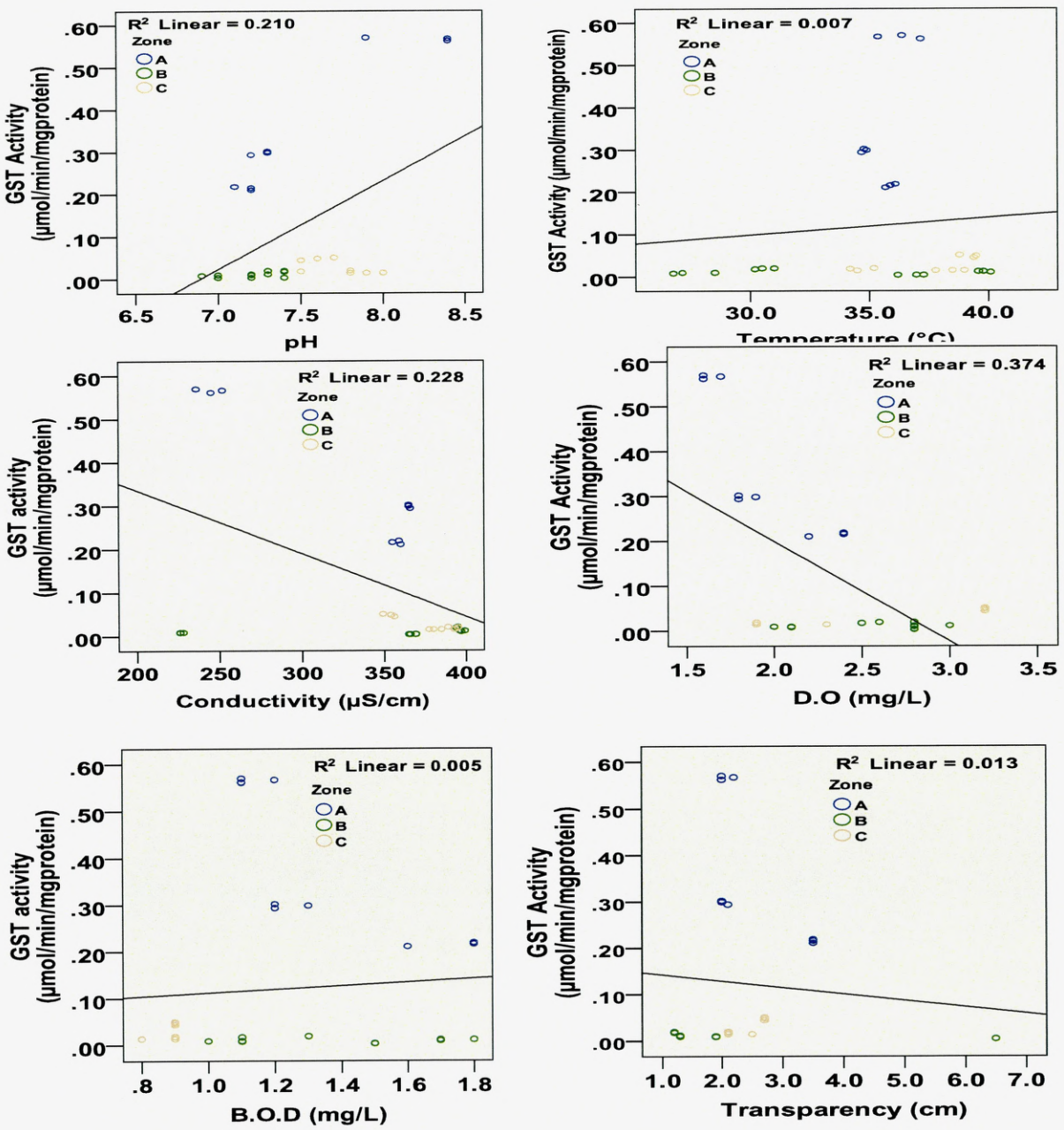

Fig. 5.4A Correlations between larval GST activity and physical environmental factors; pH, temperature, conductivity, DO, BOD and transparency. The levels of these factors and GST activity were determined, as described in Material and Methods section, in three different *An. gambiae* breeding ecologies in Northern Nigeria: Zone A, intensive agriculture; zone B, residential and zone C, petrochemical.

In contrast to P450, the chemical environmental factors; TDS, sulphates, phosphates, nitrites and nitrates showed very strong positive correlation (p= 0.000) with larval GST activity while carbon content and oil and grease displayed weak negative association (p= 0.318 and 0.063) respectively with GST activity at this life stage (Fig. 5.4B).

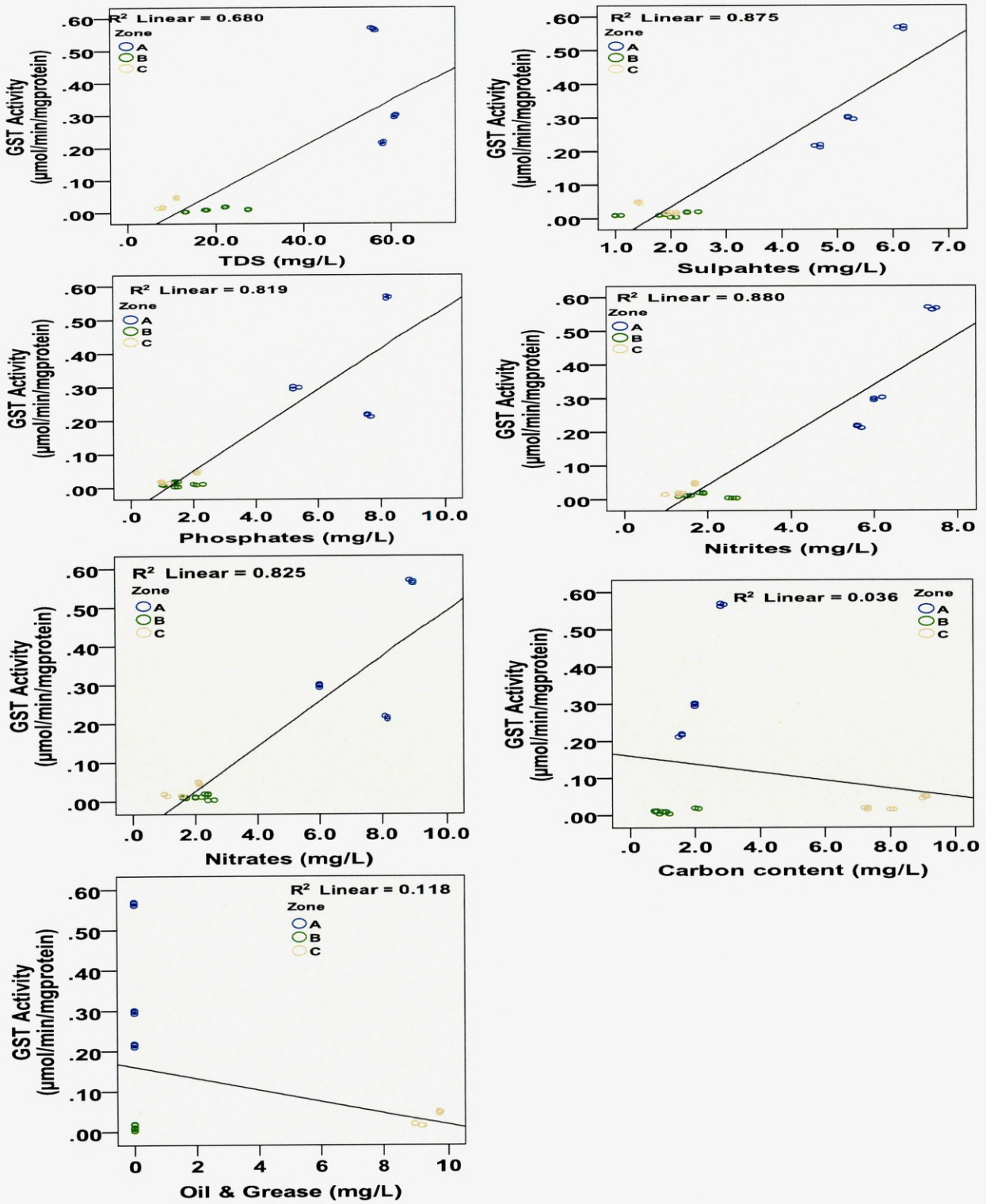

Fig. 5.4B Correlations between larval GST activity and chemical environmental factors; TDS, sulphates, phosphates, nitrites, nitrates, carbon content and oil and grease. The levels of these factors and GST activity were determined, as described in Material and Methods section, in three different *An. gambiae* breeding ecologies in Northern Nigeria: Zone A, intensive agriculture; zone B, residential and zone C, petrochemical.

ii) Pupal Stage

But for few differences, the results of the analyses on GST activity at pupal stage were very similar to those obtained in the larval stage. Both stages had similar mean GST activity distribution across the three study zones with breeding sites located in study zone A recording the highest mean activity. Also, the result of the Linear Regression Analysis were the same for both stages, with similar p-values, Coefficients of Determination (R^2) and exactly the same directions of fitted lined in their respective dot plots. Thus, the effect of each parameter on GST activity described for larval stage also applies to this stage.

iii) Adult Stage

Results obtained in this life stage were also very similar to those of the first two life stages described above.

c) α-Esterase Activity

i) Larval Stage

The result of one way ANOVA test showed that there was a highly significant differences (p=0.000) in mean α-esterase activity distribution across the three study zones with *An gambiae* from breeding sites located in study zone A recording the highest mean activity (Fig. 5.5).

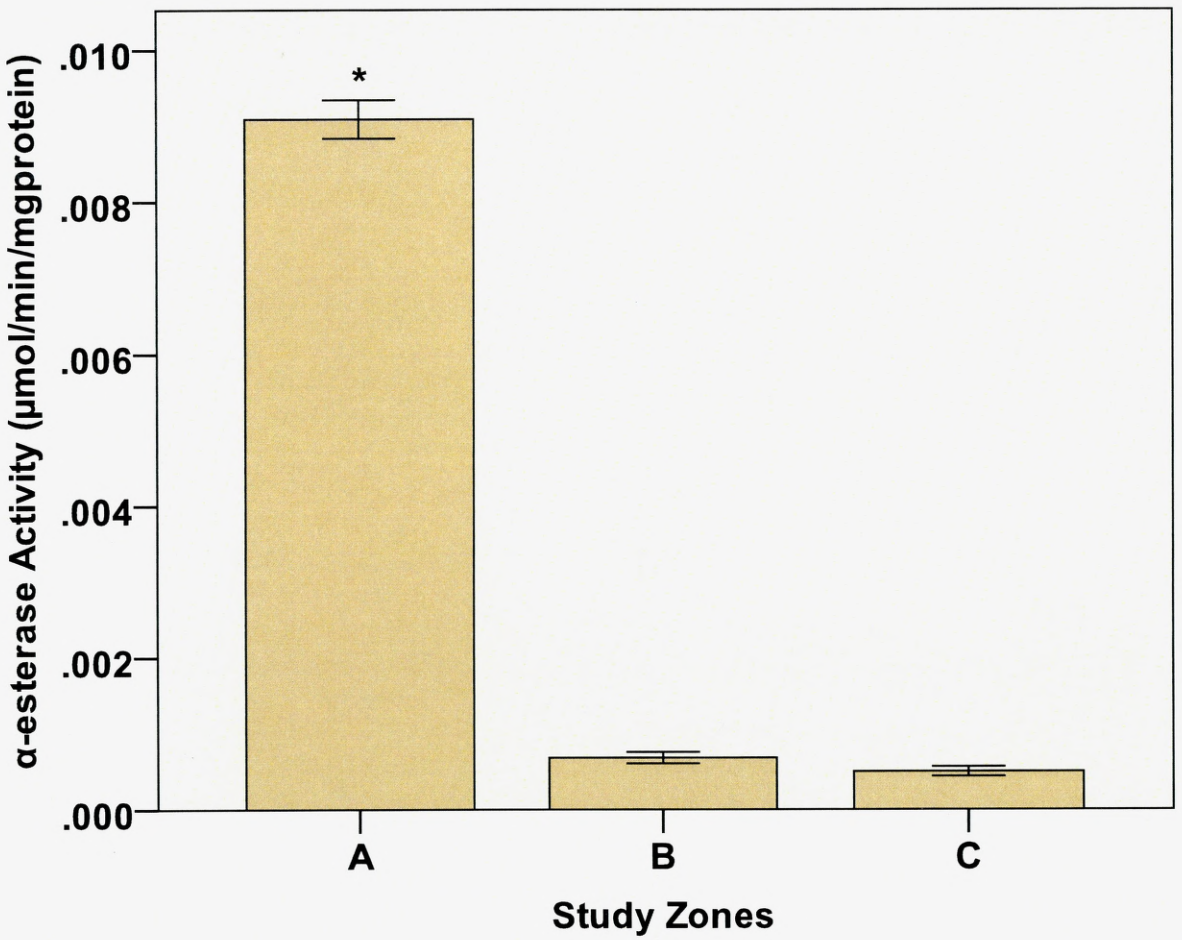

Fig. 5.5 Comparative mean distribution of α-esterase activity at the larval stage of *An. gambiae* from three different breeding ecologies in Northern Nigeria: Zone A, intensive agriculture; zone B, domestic/residential; and zone C, petrochemical. The α-esterase activity was measured using naphthyl acetate assay with 30mM acetone solution of 1-naphthyl acetate as substrate. This substrate concentration was mixed (1:10) with 0.02M phosphate buffer, pH 7.2 to produce the working solution. * indicates that the value is significantly different ($p<0.05$) from those of the other zones.

Additionally, Bonferoni Post-hoc pair-wise comparism test showed highly significant differences in mean larval α-esterase activities between zone A and B (P=0.000) and A & C (P=0.000). The difference between B and C is not significant (P=1.000).

The results of the Linear Regression analysis between each physical and chemical environmental factors and larval α-esterase activities showed that pH, temperature and transparency were not significantly associated (p= 0.382, 0.905, and 0.676) respectively, with larval α-esterase activities while conductivity, DO and BOD showed moderate correlations (p= 0.088, 0.001 and 0.184) respectively (Fig. 5.6A).

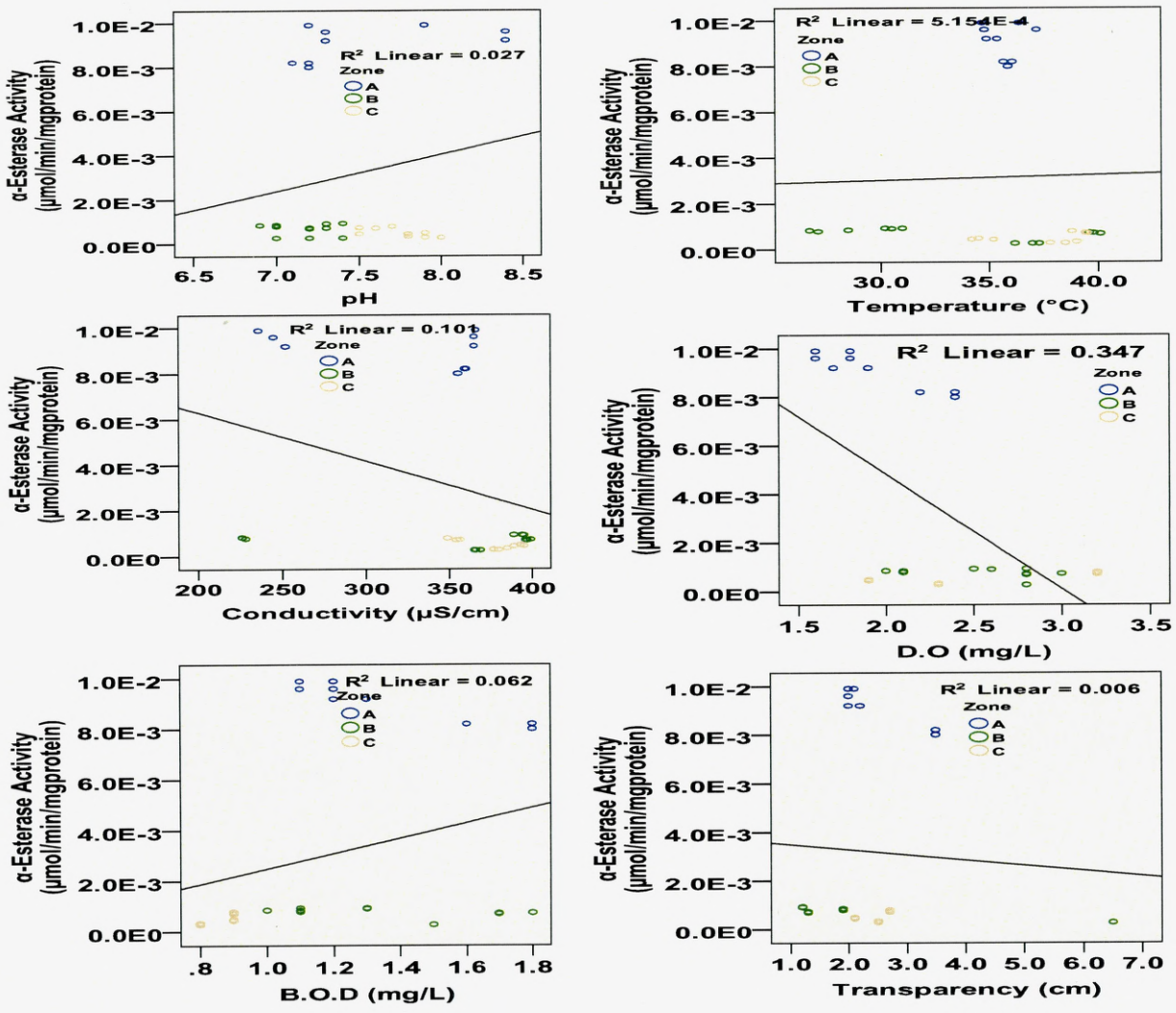

Fig. 5.6A Correlations between larval α-esterase activity and physical environmental factors; pH, temperature, conductivity, DO, BOD and transparency. The levels of these factors and α-esterase activity were determined, as described in Material and Methods section, in three different *An. gambiae* breeding ecologies in Northern Nigeria: Zone A, intensive agriculture; zone B, residential and zone C, petrochemical.

The chemical environmental parameters; TDS, sulphates, phosphates, nitrites and nitrates ions were strongly positively associated (p=0.000) with larval α-esterase activity while carbon content and oil and grease showed moderate negative correlations (p= 0.091 and 0.014) (Fig. 5.6B)

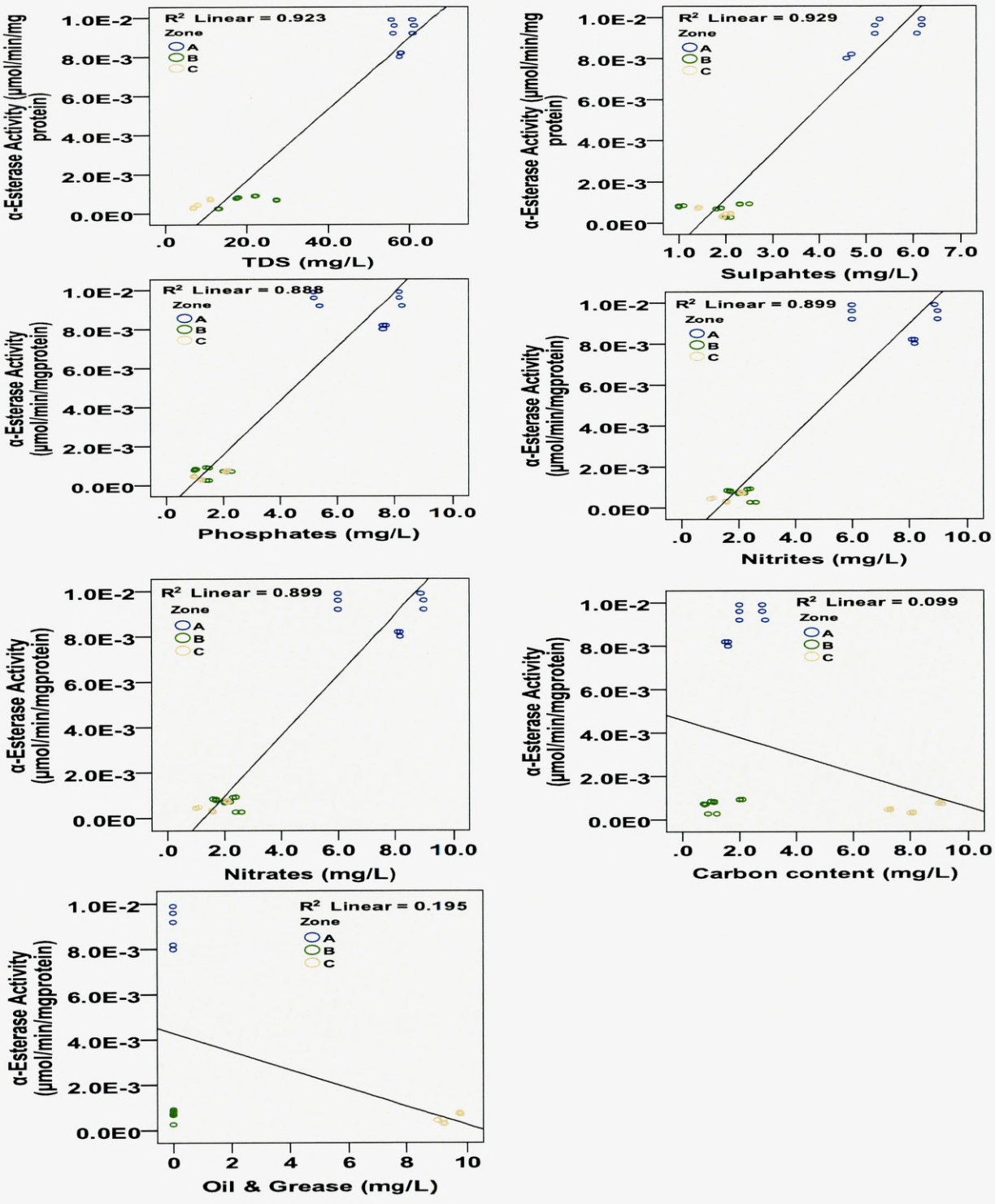

Fig. 5.6B Correlations between larval α-esterase activity and chemical environmental factors; TDS, sulphates, phosphates, nitrites, nitrates, carbon content and oil and grease. The levels of these factors and GST activity were determined, as described in Material and Methods section, in three different *An. gambiae* breeding ecologies in Northern Nigeria: Zone A, intensive agriculture; zone B, residential and zone C, petrochemical.

ii). Pupal Stage

The observations recorded at the pupal stage were very much similar to those obtained for the larval stage. The mean activity distribution of α-esterase activity at this stage was similar to that of the larval stage with study zone A accounting for the highest mean activity. Also, the result of the Linear Regression analysis was the same for both stages with similar p-values, coefficient of determination(R^2), and exactly the same direction for the fitted lines in their respective dot plots. Thus, the effect of each of the environmental parameters on α-esterase activity as described for the larval stage also applies to this stage.

iii). Adult Stage

The observations recorded at this stage were also similar to those recorded at the larval and pupal stages of *Anopheles gambiae* described above.

d). β-Esterase Activity

i) Larval Stage

The result of one way ANOVA test carried out to examine the differential mean activity distribution of *An. gambiae* β-esterase at the larval stage of development showed that there was a highly significant differences (p=0.000) in mean activity distribution across the three studied zones with breeding sites located in zone A (as in α-esterase), accounting for the highest activities (Fig. 5.7).

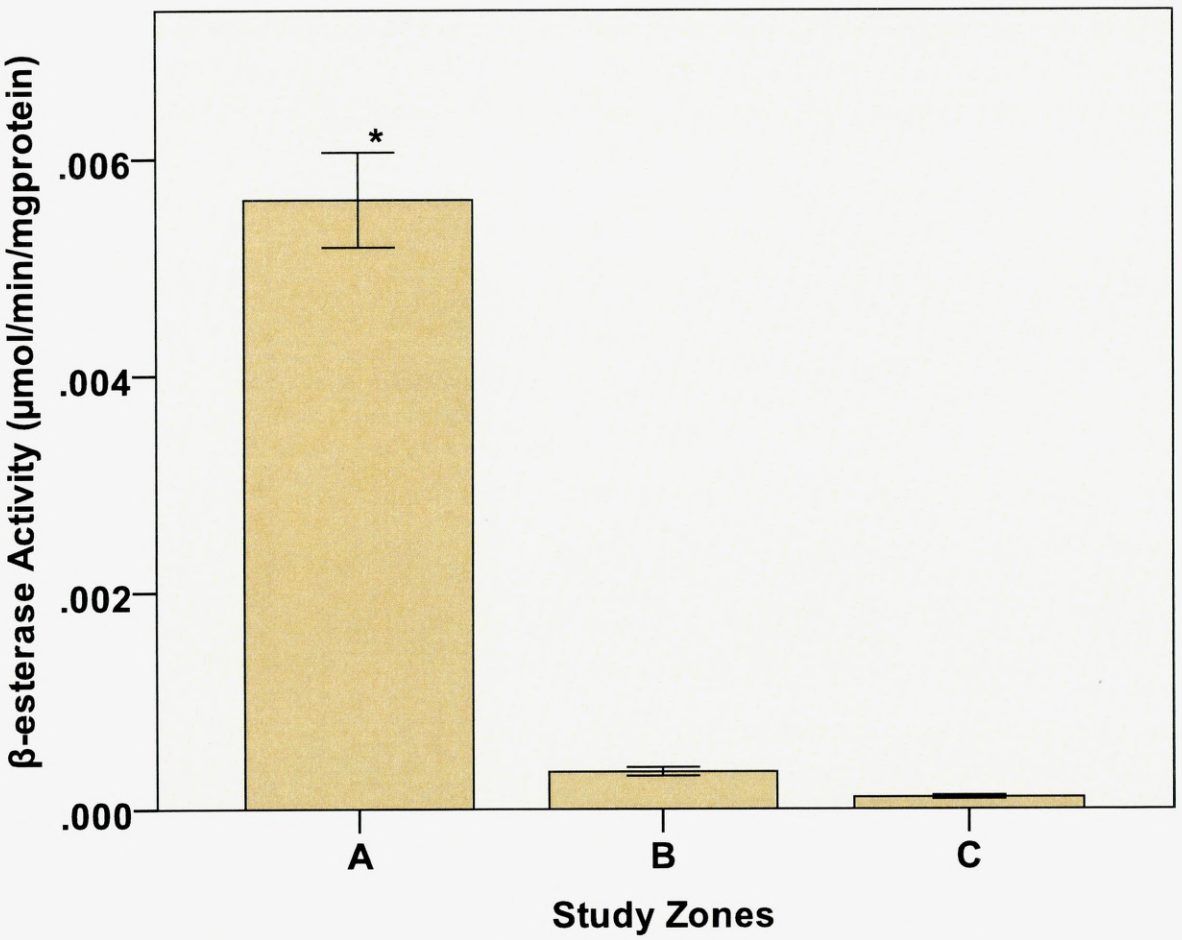

Fig. 5.7 Comparative mean distribution of β-esterase activity at the larval stage of *Anopheles gambiae* from three different breeding ecologies in Northern Nigeria: Zone A, intensive agriculture; zone B, domestic/residential; and zone C, petrochemical. The α-esterase activity was measured using naphthyl acetate assay with 30mM acetone solution of 2-naphthyl acetate as substrate. This substrate concentration was mixed (1:10) with 0.02M phosphate buffer, pH 7.2 to produce the working solution. * indicates that the value is significantly different ($p<0.05$) from those of the other zones.

As was recorded for α-esterase enzyme, Pairwise comparism test showed that the differences in mean activity distribution between zone A & B and A & C were highly significant (p=0.000) while that between B & C was not (p=1.000).

The data in Figures 5.8A & 5.8B illustrates the results of Linear Regression Analyses, carried out to examine the effect of the individual environmental factors on the activities of β-esterase enzyme at the larval stage of *An. gambiae*. According to this results, pH, temperature, BOD and transparency were also not significantly associated (p= 0.337, 0.977, 0.301, and 0.576) respectively, with larval β-esterase activity (similar to that recorded for α-

esterase) while conductivity and DO showed moderate negative association (p= 0.099 and 0.000).

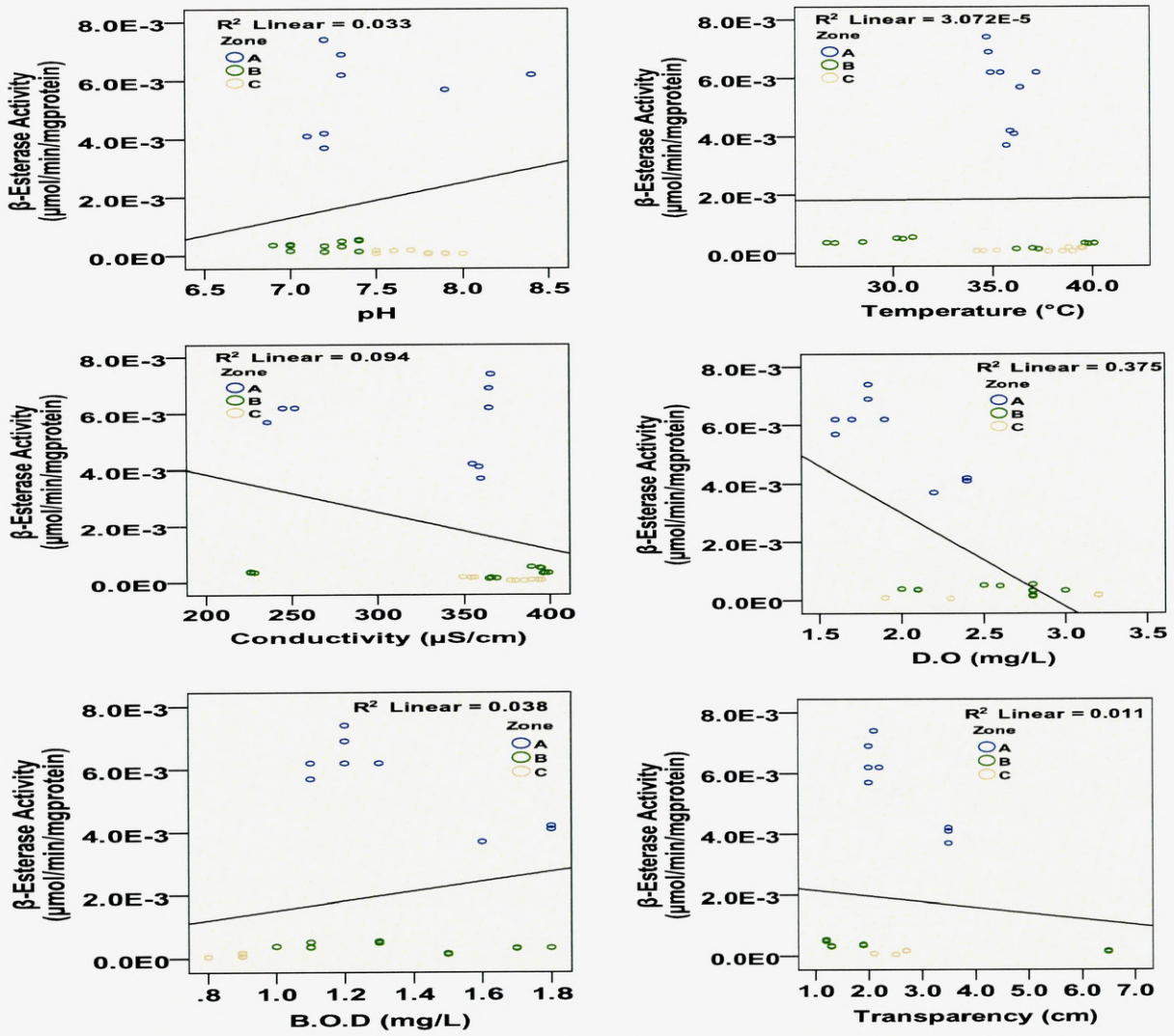

Fig. 5.8A Correlations between larval β-esterase activity and physical environmental factors; pH, temperature, conductivity, DO, BOD and transparency. The levels of these factors and α-esterase activity were determined, as described in Material and Methods section, in three different *An. gambiae* breeding ecologies in Northern Nigeria: Zone A, intensive agriculture; zone B, residential and zone C, petrochemical.

However, similar to α-esterase enzyme, the chemical environmental factors (i.e. TDS, sulphates, phosphates, nitrites, and nitrates) produced a strong positive association (p= 0.000) with larval β-esterase activity, while carbon content and oil and grease were also moderately

negatively associated (p= 0.088 and 0.013) respectively, with *An. gambiae* larval β-esterase activity.

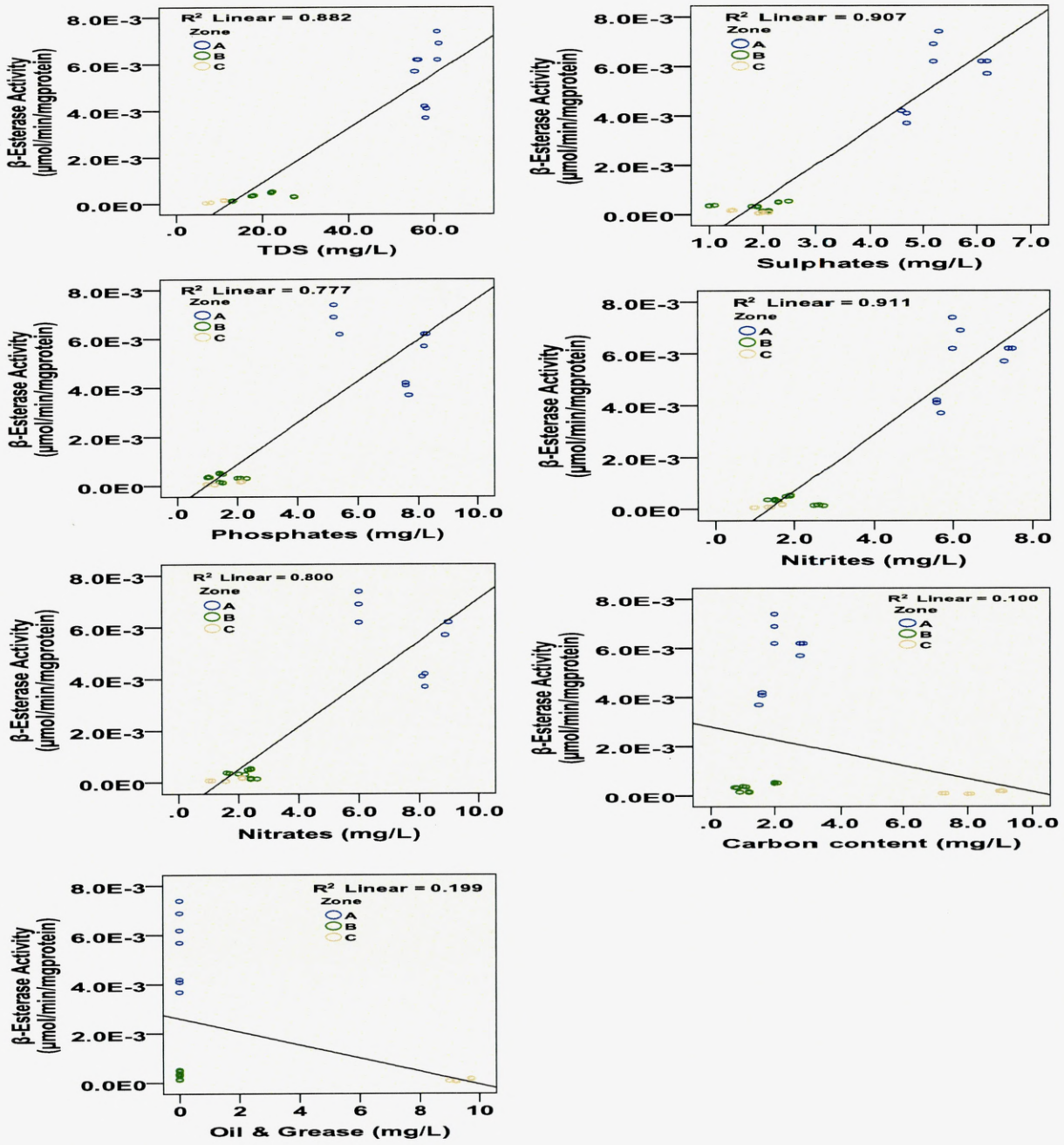

Fig. 5.8B Correlations between larval β-esterase activity and chemical environmental factors; TDS, sulphates, phosphates, nitrites, nitrates, carbon content and oil and grease. The levels of these factors and GST activity were determined, as described in Material and Methods section, in three different *An. gambiae* breeding ecologies in Northern Nigeria: Zone A, intensive agriculture; zone B, residential and zone C, petrochemical.

ii) Pupal Stage

All the results of the analyses at this life stage also produced similar outcomes as in the larval stage. The distribution of the mean enzyme activity across the three study zones were similar to that of the larval stage, with study zone A recording the highest mean activity. Also, the outcomes of the Linear Regression analysis were similar to those of the larval stage.

iii). Adult Stage

Lastly, similar analyses were also carried out at the adult stage of *An. gambiae* and the results and observations were also very much similar to those obtained in the two preceding stages i.e. larval and pupal stages.

5.2.2.2 Effect of the Physico-chemical environmental Factors on the Activities of Detoxification Enzymes.

In order to deduce a statistical model showing a combination of the physico-chemical environmental variables that produce the most combined significant effect on the detoxification enzymes, factor analysis was carried out on the detoxification enzymes followed by redundancy analysis between the extracted principal components of the detoxification enzymes and those of the physico-chemical environmental variables. As explained earlier (Chapter 4; Section 4.2.2.3), classical multivariate regression between the environmental variables and the detoxification enzymes failed to produce a reliable model estimates due to strong colinearity among the physico-chemical variables as well as among the detoxification enzymes. Therefore factor analysis was employed to extract components from both the physico-chemical variables (Chapter 4 Section 4.2.2.3) and the detoxification enzymes.

The result of the factor analysis carried out on the detoxification enzymes produced three extracted principal components which explained 99% of the variability in the data (See Appendix for both Total Variance and Rotated Component Matrix). According to the factor loading the first principal component (PC 1) correlates strongly with α and β-esterase activities and some elements of GST, PC 2 correlated with the P450 enzymes activities whereas PC 3 is associated with GST alone. Therefore, according to the results of the factor analysis, these three principal components explained more than 99% of all the variability in the detoxification enzymes irrespective of life stage. As shown in the Scree plot of the extracted principal components (Fig. 5.9A), these three components were distinctly separated from the remaining factors or components that explained little or nothing about the original detoxification enzyme variables.

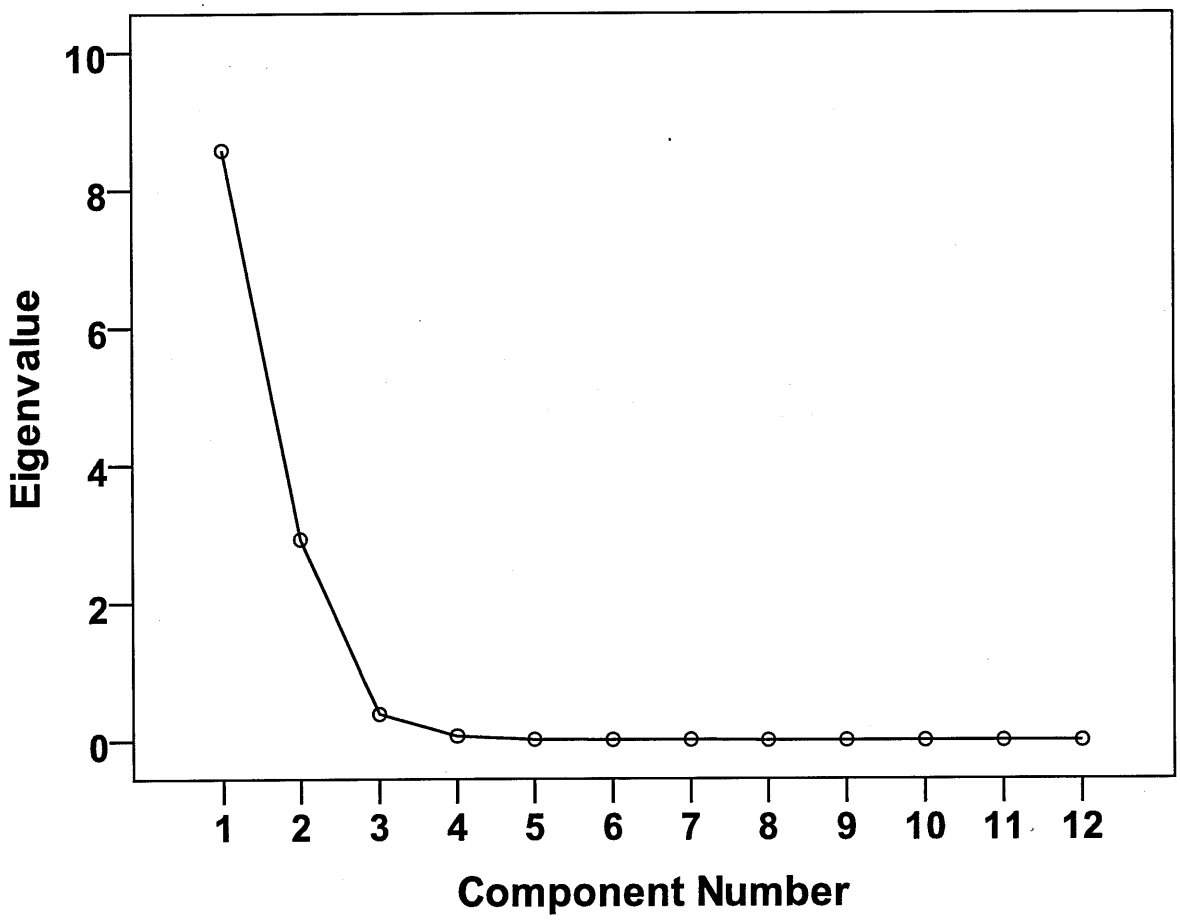

Fig. 5.9A Scree Plot of the extracted components from factor analysis of the detoxification enzyme variables. Components 1-3 explained 99% of the variability in the data.

Furthermore, a scatter plot (Fig. 5.9B) of the scores of the two of the extracted components showed that the data points cluster very well according to the three study zones were the data originated from.

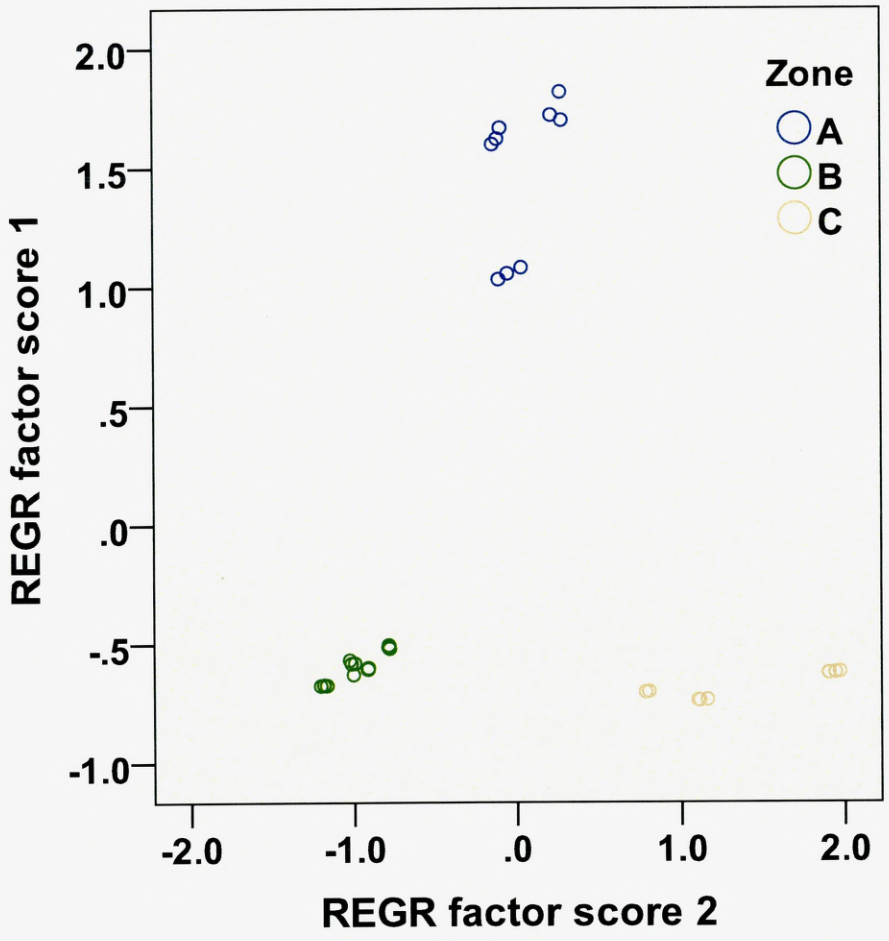

Fig. 5.9B Scatter plot of the scores of factor 1 and 2. Factor 1 (component 1) represents GST and α & β-esterases across the three life stages while factor 2 (component 2) represents P450 activities across the three life stages of *Anopheles gambiae*. Zone A; intensive agricultural areas, Zone B; Domestic/residential environments, Zone C; Petrochemical/hydrocarbon laden.

Finally, Redundancy analysis was carried out between the extracted principal components of the physico-chemical environmental variables (Table 4.5) and the extracted principal components of the detoxification enzymes (PC 1-3). The result of redundancy analysis between the principal components extracted from the physico-chemical environmental variables and GST and α and β-esterases enzymes (i.e. PC 1 of the detoxification enzyme variables; Fig. 5.9A) is presented in Table 5.5 below. According to this result (Table 5.5), all pesticide and fertilizer contaminants, the petrochemical/hydrocarbon contaminants and only the physical environmental factors; conductivity, transparency, DO and BOD produced the

most combined significant effect on the activities of these two enzymes (i.e. GST and α and β-esterases).

Table 5.5 Physico-chemical Environmental Factors or Components with Combined Effect on GST and α & β-esterases across the three Life Stages of *An. gambiae* .

			95% Wald Confidence Interval		Hypothesis Test		
Parameter	**B**	**Std. Error**	**Lower**	**Upper**	**Wald Chi-square**	**df**	**Sig.**
Intercept	-8.072E-016	0.0194	-0.038	0.038	0.000	1	1.000
PC 1[a]	0.955	0.0198	0.916	0.994	2334.535	1	0.000
PC 2	-0.109	0.0198	-0.148	-0.071	30.603	1	0.000
PC 3	-0.106	0.0198	-0.144	-0.067	28.561	1	0.000
PC 4	-0.010	0.0198	-0.049	-0.029	0.256	1	0.607
PC 5	-0.070	0.0198	-0.109	-0.032	12.720	1	0.000
PC 6	-0.196	0.0198	-0.235	-0.157	98.394	1	0.000
PC 7	-0.006	0.0198	-0.044	-0.033	0.081	1	0.776
PC 8	-0.099	0.0198	-0.138	-0.061	25.297	1	0.000
Scale	0.011[b]	0.0029	0.007	0.019			

[a]: Refer to Table 4.5

[b]: Maximum likelihood estimates

Since some components of GST were also extracted in the detoxification enzymes factor analysis as PC 3 (See Appendix for both Total Variance and Rotated Component Matrix) in addition to that contained in PC 2, redundancy analysis involving these components of GST and the physico-chemical environmental factors showed that in addition to the parameters that produced effect on the combined GST and α and β-esterases enzymes (Table 5.5), pH also produced an effect on GST activities.

Lastly, the result of the redundancy analysis (Table 5.6) between the extracted components of the environmental physico-chemical factors (Table 4.5) and P450 activities (i.e. PC 2 of the detoxification enzyme variables; Fig. 5.9A) showed that, unlike GST and α & β-esterases enzymes, all the eight extracted components of the physico-chemical environmental variables (Table 4.5) produced a combined effect on P450 activities. Thus pesticide and fertilizer contaminants, petrochemical contaminants and the physical environmental factors; pH, temperature, conductivity, transparency, DO and BOD all produced a combined effect on the activities of P450 across the three life stages of *An. gambiae* from Northern Nigeria.

Table 5.6 Physico-chemical Environmental Factors or Components with Combined Effect on P450 Activities across the three Life Stages of *An. gambiae* .

			95% Wald Confidence Interval		Hypothesis Test		
Parameter	**B**	**Std. Error**	**Lower**	**Upper**	**Wald Chi-square**	**df**	**Sig.**
Intercept	-7.181E-016	0.0073	-0.014	-0.014	0.000	1	1.000
PC 1[a]	0.143	0.0074	0.128	0.157	373.714	1	0.000
PC 2	0.940	0.0074	0.925	0.954	16178.348	1	0.000
PC 3	0.038	0.0074	0.023	0.052	25.838	1	0.000
PC 4	0.233	0.0074	0.218	0.247	993.202	1	0.000
PC 5	-0.093	0.0074	-0.108	-0.079	159.984	1	0.000
PC 6	0.138	0.0074	0.062	0.091	350.375	1	0.000
PC 7	0.076	0.0074	0.061	0.090	107.203	1	0.000
PC 8	0.075	0.0074	0.061	0.090	103.993	1	0.000
Scale	0.002[a]	0.0004	0.001	0.003			

[a]: Refer to Table 4.5

[b]: Maximum likelihood estimates

5.3 Discussion

This study demonstrated a significant variation in mean activity distribution of the three detoxification enzymes (i.e. GST P450 and α & β-esterases) assayed in *An. gambiae* sampled from many breeding sites across the three studied zones. The highest mean P450 activities was recorded in *An. gambiae* sampled from breeding sites located in study zone C (Figure 5.1) while the lowest mean P450 activity was recorded in study zone B (residential/domestic areas). *An. gambiae* from breeding sites located in zone A (intensive agricultural areas) also recorded significant mean P450 activity. Statistical analysis showed that the difference in mean activity distribution between zones A&C was not highly significant ($p=0.160$) while the differences between zones A&B and B&C were highly significant ($p=0.008$ and 0.000) respectively. Analysis of the differential distribution of the levels of the environmental chemical factors showed that breeding sites located in zone C accounts for the highest levels of carbon content and oil and grease. The levels of the other chemical factors (sulphates, phosphates, nitrite and nitrate ions) were highest in breeding sites located in study zone A. The result of Linear Regression analysis carried out between these environmental factors and *An. gambiae* P450 activities showed that TDS, sulphates, phosphates, nitrite and nitrate ions produced a low to moderate significant association ($p<0.05$) with P450 activities across the three life stages of *An. gambiae* studied. However, carbon content and oil and grease produced a strong positive correlation ($p<0.05$) with P450 activities (Figure 5.2B). This means that among the chemical environmental factors studied, carbon content and oil and grease appeared to produce the most significant effect on the activities of P450. Additionally, among the physical environmental factors studied, pH, temperature and BOD significantly influenced P450 activities while conductivity, DO and transparency appeared to produce little or no significant ($p<0.05$) association (Fig. 5.2A). However, the results of the Factor, followed by Redundancy analysis produced a model containing all the chemical

environmental factors (pesticides and fertilizer contaminants, and petrochemical contaminants) together with the physical environmental factors; pH, temperature, conductivity, transparency, DO and BOD as producing the most singular combined effect on *An. gambiae* P450 activities irrespective of the life stage. Thus, the outcome of the factors and redundancy analysis augmented and complemented that of linear regression in deducing a model of physico-chemical environmental factors that affected the activities of P450 in *An. gambiae*.

Furthermore, this study revealed that the activities of GST and α and β-esterases produced the same observations in their individual relationships with the environmental factors. The highest mean activities of these two detoxification enzymes (GST α and β-esterases) were recorded in breeding sites located in study zone A (Figure 5.3, 5.5 and 5.7). Also, statistical analyses showed highly significant differences in mean activity distribution of these enzymes between zone A & B and A & C ($p=0.000$), while that between B & C were not significant ($p=1.000$). These observations were recorded across the three life stages of *An. gambiae* studied. This means that *An. gambiae* sampled from breeding sites located in intensive agricultural areas (zone A), with the highest levels of sulphate, phosphate, nitrite and nitrate ions, brought into the mosquito breeding water by runoffs from surrounding farmlands, where NPK fertilizer, organic manure and several types of agro-pesticides are used, recorded the highest mean activities of these two detoxification enzymes (GST and α & β-esterases). Additionally, Linear Regression analyses between the levels of the environmental chemical factors and activities of these enzymes showed that sulphate, phosphate, nitrite and nitrate ions produced a strong positive association ($p<0.05$) with GST and α & β-esterases, while carbon content and oil and grease showed a weak positive association (Figure 5.4B, 5.6B and 5.8B). This means that as the levels of these factors increases the activities of GST and α & β-esterases also increases. Also, Linear Regression with the physical environmental factors

produced similar observation in both enzymes, with pH, conductivity and DO showing moderate association while temperature, transparency and BOD showing little or no significant($p<0.05$) association (Fig. 5.4A, 5.6A and 5.8A). Additionally, Factors analysis extracted both detoxification enzymes (GST and α & β-esterases) across the three life stages as a single principal component (Fig. 5.9A) and a model was deduced (Table 5.5) using Redundancy analysis, which selected pesticide and fertilizer contaminants, petrochemical contaminants, as well as the physical environmental factors; conductivity, transparency, DO, and BOD as producing the most singular combined effect on the activities of these detoxification enzymes.

Inductive responses of detoxification systems to xenobiotic overload have been well documented in several organisms including insects. Induction of detoxification enzymes by up-regulation of the genes responsible for their synthesis is one major mechanism employed by organisms to respond to exposure to high levels of environmental chemicals and a number of transcriptions factors regulating this mechanism have been documented in many organisms (Misra *et al.*, 2011). Induction of detoxification enzymes in response to environmental xenobiotics have also been documented in insects. The first documented evidence of enzyme induction in insects was given by Agnosin and Dinamarca, in which they reported an increased activity of NAD kinase in *Triatoma infestans* after exposure to DDT (Agnosin and Dinamarca, 1963). Cytochrome P450s have featured more prominently in many previous studies involving induction of detoxification enzymes in response to environmental xenobiotics in insects, relative to GST and α and β-esterases. Le Goff (2006) demonstrated the induction of multiple P450 genes, including those previously implicated in insecticides resistance, after exposure of *Drosophila* to chemical contaminants such as Phenobarbital and atrazine. In general, induction of detoxification enzymes in response to several xenobiotic exposures in many insect species have been well documentwd (David *et al.*, 2013). However,

no studies have, to my knowledge, demonstrated the inductive capacity of the environmental chemical species considered in this study on the activities of detoxification enzymes in *An. gambiae* in Northern Nigeria, despite considerable evidence which indicate that *Anopheles* mosquitoes thrives in breeding ecologies where they could be exposed to these environmental chemicals.

The aim of this present study is to establish the potentiality of different physico-chemical environmental factors as driving a selection pressure for the emergence and development of insecticides resistance in *An. gambiae*. This is because, as stated earlier (Chapter 3), of the similarity in structures, functions and activity relationships between these environmental factors and several synthetic insecticides used in mosquito control. The hypothesis here is that prior exposure of products containing these chemical species present in *An. gambiae* breeding ecologies, could exerts a selection pressure that could drive an intrinsic and acquired capacity in *An. gambiae* towards tolerance to several types of insecticides used for its control. One of the major mechanisms for the development of insecticide resistance in mosquitoes is detoxification enzymes mechanism. This involve increase in the activities of detoxification enzymes (P450, GST α and β-esterases) which lead to rapid metabolism of the insecticides before it reaches its sites of action (Hemingwy *et al.*, 2004; David *et al.*, 2013). Therefore, exposure of *An. gambaie* to different environmental chemical compounds presents in its breeding habitats, which could induce increase in activities of these enzymes could potentially produce intrinsic and acquired tolerance to insecticides in mosquitoes emerging from such breeding ecologies, especially if these chemical compounds possess similar structures and activity relationship with the various insecticides used in mosquito control.

A comparative analysis between activities of the detoxification enzymes recorded in this study and those of *An. gambiea* displaying metabolic resistance to DDT and pyrethroids insecticides in Nigeria's West African neighbours; Burkina Faso and Cameroun was carried

out. Previous studies carried out by Namountougou *et al.*, (2012), Nwane *et al.,* (2013) and Etang *et al.*, (2007) in Burkina Faso and cameroun had demonstrated DDT and pyrethroids metabolic resistance in *An. gambiae*. In these studies, activities of the three major detoxification enzymes were implicated, among other mechanism, as conferring resistance in their resistant strain of *An. gambiae* in comparism to the Kisumu strain which was used as reference susceptible standards in all of these studies. Comparing the detoxification enzyme activities of the resistant and susceptible reference Kisumu strains reported in these previous studies with the activities recorded in this present study showed that P450 and GST activities favourably compared, and even in many cases, higher than those reported in Burkina Faso and Cameroun resistant strains (Namountougou *et al.*, 2012; Nwane *et al.*, 2013; Etang *et al.*, 2007). However, α-esterase activities recorded in this present study was lower than those from these previous studies. Interestingly, most of the lowest P450 and GST activities recorded in this study were higher than those of the Kisumu susceptible reference standards used and reported in the Burkina Faso and Cameroun studies (Namountougou *et al.*, 2012; Nwane *et al.*, 2013; Etang *et al.*, 2007). While this comparative analysis was not intended to indicate that the *An. gambiae* samples in this study were also resistant to these insecticides, the result nevertheless serve to establish comparism with strains of *An. gambiae* confirmed to be displaying metabolic resistance to various insecticides through the activities of these detoxification enzymes. However, the results suggest that the population of mosquitoes in some of these breeding ecologies studied in Northern Nigeria may have developed or are selectively being primed to develop resistance to insecticides.

This study thus revealed that *An. gambiae* emerging from breeding sites located in study zone A and C could be selected for potential tolerance to insecticides, especially those having similar structures and activity relationship to the environmental chemical compounds present in high levels in these breeding sites. Studies from previous studies have demonstrated the

contribution of prior exposure to various environmental xenobiotics to the development of insecticides resistance by several insect species. Boyer *et al.*, (2006) reported that *Aedes aegypti* larvae exposed to the herbicides atrazine became more tolerant to the organophosphate temephos. Similarly, exposure of *Aedes albopictus* larvae to benzothiazole and pentachlorophenol increased their tolerance to insecticides such as cabaryl, rotenone, and temephos (Suwanchaichinda and Brattsen, 2001; 2002). In addition, other studies have established a correlation between increase in tolerance to insecticides in many insects and induction of detoxification enzymes as a result of prior exposure to environmental xenobiotics (Feyereisen, 2005; Hemingway *et al.*, 2002; 2004). Furthermore, finding from studies by Poupardin *et al.*, (2008) further highlighted the contribution of prior exposure of mosquitoes to environmental xenobiotics to the development and emergence of insecticides resistance. In this study, *Aedes aegypti* larvae were exposed to sub-lethal concentrations of three different xenobiotics likely to be found in highly polluted breeding sites. These xenobiotics include the herbicide atrazine, the polycyclic aromatic hydrocarbon fluoranthene and the heavy metal copper. The larvae were then exposed to two chemical insecticides; the organophosphates temephos and the pyrethroids permethrin. Larval tolerance to the insecticides and detoxification enzymes were compared. The results showed a marginal increase in tolerance to the insecticides in the presence of the xenobiotics and the discussion suggests that the phenomenon might even be more pronounced in highly polluted breeding sites or following temporary dramatic pollution events (Poupardin *et al.*, 2008). While many of these previous studies established relationship between exposures to some environmental xenobiotics and incidence of insecticides resistance in various mosquito species, this present study was conducted at the level of pre-insecticide exposure to implicate some broad-base human activities, such as those described in this study, as potentially driving intrinsic and acquired tolerance to insecticides in *An. gambiae* in Northern Nigeria. Moreover, *An.*

gambiae has not featured prominently in many of these previous studies and none of these studies have to my knowledge been carried out in Northern Nigeria. Thus, this study became necessary in view of the fact that *An. gambaie* is the major malaria vector in Nigeria (Okwa, Akinmolayan, Carter and Hurd, 2009) and Nigeria accounts for the highest malaria deaths in Sub-Saharan Africa (WHO 2010).

Findings from this study were also consistent with observations from several previous studies which implicated agricultural practices as a selection factor in the development and emergence of insecticides resistance in various insect species from many other parts of the world. For instance, Georghiou *et al.*, (1982) demonstrated organophosphate resistance in *An. albimamus* following intensive treatment of cotton pest with pesticides in El-Salvador. The resurgence of malaria in India and Central America was linked to intensive agricultural production employing intensive use of agricultural pesticides (Chapin and Wasserstrom, 1981). Brogdon *et al.*, (1988) demonstrated elevated levels of acetylcholinesterase activity in *An. albimamus* in intensively managed agricultural areas in Guatemala. Furthermore, a comparative analysis involving two malaria vectors; *An. nigerrimus* and *An. culcifaciens* was carried in Sri Lanka. The former breeds in intensive agricultural areas while the latter breeds in non agricultural areas. The results of the analysis showed that *An. nigerrimus* was resistant to organophosphate and carbamates at both larval and adult stages while *An. culcifaciens* was not. This established the role of agriculture as source of selection pressure for development of resistance in *An. nigerrimus* (Hemingway *et al.*, 1986). These and other similar studies have established the impact of agricultural practices in the emergence and development of insecticides resistance. Majority of these studies focused primarily on the role of the use of agricultural pesticides as selection factor in the development of resistance to public health insecticides. But other agrochemicals other than pesticides, such as fertilizer studied here, could also play an important role. In addition, most of these previous studies were conducted

at the level of post-insecticides exposure. However, similar studies carried out at the level of pre-insecticides exposure are necessary in order to evaluate the extent and nature of the role of various agricultural practices as selection pressure for the development and emergence of insecticides resistance in public health vectors. Thus, this present study, which to my knowledge is the first of its kind in Northern Nigeria, aim to bridge this gap by assessing the importance of two major agricultural practices; pesticide and fertilizer application, as potential sources of selection pressure for the development and emergence of insecticides resistance in *An. gambiae* in Northern Nigeria.

This study revealed that *An. gambiae* larvae thrives in breeding sites laden with petrochemical/hydrocarbon products. Larval prospecting and collection was conducted in these habitats. Though, larval density was lower compared to breeding sites located in intensive agricultural and residential areas (Table 2.4), appreciable amount of *An. gambiae* larvae were nevertheless collected from these breeding sites. Biochemical enzyme analysis of *An gambiae* mosquito collected from these breeding sites revealed that the activities of P450 monooxygenase enzyme was significantly higher in these petrochemical laden breeding zone relative to the other two studied zones (zone A and B). Statistical analysis also showed that carbon content and oil and grease; levels of which were significantly higher in breeding sites located in this zone compared to intensive agriculture and residential zones were strongly positively associated with P450 activities. The model deduced from redundancy analysis for the combined effect of physico-chemical factors on P450 activities also prominently selected carbon content and oil and grease as producing the most significant influence on the activities of P450 across the three life stages of *An. gambaie*. Thus, these observations showed that petrochemicals or hydrocarbon products could induce significant P450 activities in *An. gambiae*. Although, this study could not conduct elaborate hydrocarbon profile of these

breeding sites, therefore, it is not clear which specific hydrocarbon specie is responsible for the observed inductive effect.

The effect of the presence of petrochemical/hydrocarbon products on the growth, survival and biochemical behaviour of *An. gambiae* has not, to my knowledge, been largely investigated, despite an age long tradition of applying these products to mosquito breeding waters to control mosquito larvae (Burton, 1967; Rozendaal, 1997)). Findings from previous studies (Ekom, 2006; Obire and Anyawun, 2009; Adekunle *et al.*, 2010; Patrick-Uwanyanwu *et al.*, 2011) have however demonstrated several effects of petrochemical products on many other aquatic organisms in Nigeria. The induction of cytochrome P450 systems in response to exposure of insects to petrochemical products has not been largely investigated. However, since this enzyme system together with the other detoxification machinery are also conserved in insects (Strode *et al.*, 2008)), inferences from findings in other organisms can be used to explain the observations made in this present study.

Furthermore, studies have demonstrated correlations between P450 activities and levels of fluorescent aromatic compounds in some marine organisms following oil spill. In one such studies, fished collected from marine environments contaminated by oil spill were found to display higher P450 activities which correlates with levels of polycyclic aromatic hydrocarbons (Lee and Anderson 2005). Evidence of P450 induction and increased levels of bile fluorescent aromatic hydrocarbons in birds and mammals have also been documented (Lee and Anderson, 2005 David *et al.*, 2013)). Finally, several studies on the mechanisms of induction involving detoxification enzymes have identified hydrocarbon activated receptors in the regulation of induction of detoxification enzymes including P450. Misra *et al.*, (2011) demonstrated the role of transcription factors such as aryl hydrocarbon receptors (AHR) and aryl nuclear translocator (ARNT) in the regulation of detoxification genes in response to environmental xenobiotics. Specifically, petrochemical compounds such as 2,3,7,8-

tetrachlorodibenzene-*p*-dioxin and polycyclic aromatic hydrocarbon have been established as activators of these transcription factors (Schecter, *et al.*, 2006).

This present study demonstrated increase in the activities of P450 enzyme in response to exposure of *An. gambiae* mosquito to petrochemical products, and the increase in activity correlated with levels of these products. Previous findings on insecticides resistance in several species of insects have also demonstrated increased activities of P450 systems as one of the major mechanisms of resistance to several synthetic insecticides. For instance Vulule *et al.*, (1999) demonstrated increased heme levels of P450 cytochromes in pyrethroid resistant population of *An. gambiae* in Kenya. This was followed in 2003 that CYP6Z1 was overexpressed in a pyrethroid resistant *An. gambiae* , giving an indication of the sub family of P450 involved in pyrethroid resistance (Nikou *et al.*, 2003). Awolola *et al.*, (2009) reported constitutive over expression of several P450 families in permethrin resistant *An. gambiae* in Nigeria. Several of these P450 families implicated in pyrethroid resistance were shown to possess broad spectrum substrate specificity and could be involved in the metabolism of several other xenobiotics other than insecticides (McLaughlin *et al.*, 2008). In addition, several families of P450 cytochromes were reported to be over expressed in DDT resistant strains of *An. gambiae* (David *et al.,* 2005; Himeidan *et al.*, 2007). Thus, these and other similar studies have established over expression of P450, as well as other detoxification enzymes, as a major mechanism of insecticides resistance in insects. However, most of these studies were conducted after insecticides exposure and the role environmental xenobiotic which their strains of mosquitoes may have encountered in their breeding ecologies, prior to exposure to insecticides was not taken into account. This present study was conducted at pre-insecticide exposure level, and the results showed that *An. gambiae* emerging from some specific breeding sites, could already have acquired an intrinsic enzymatic machinery that could allow it to tolerate various synthetic insecticides even prior to exposure. This study thus

demonstrated that *An. gambiae* emerging from petrochemical/hydrocarbon contaminated breeding sites could be potentially selected for tolerance to insecticides, especially those insecticides whose major routes of metabolism involve the P450 systems.

Finally, the complete detoxification or metabolism of most environmental xenobiotics and/or their constituent chemical species, require the activities of several other biomolecules or cofactors other than the three major detoxification enzymes. Glutathione is one of the most important of such cofactors. Apart from serving as substrate to some of the detoxification enzymes (e.g. GST), it also takes part in the direct conjugation of several xenobiotic compounds. Therefore, it is important to assess the contribution of glutathione to the overall detoxification process and to examine its relationship or association with the detoxification enzymes in *An. gambiae*. This could provide vital information on the regulation of synthesis and activities of these detoxification enzymes, a phenomenon that has been identified as one of the major approach for managing insecticides resistance.

5.4 Conclusion

This study has demonstrated the significance of the physico-chemical environmental factors present in mosquito breeding sites, not only on the growth, development and survival of *An. gambiae*, but also on their detoxification enzymes machinery. Significant associations were established between several physico-chemical environmental factors and activities of three major detoxification enzymes P450s, GSTs and CEs) in *An. gambiae*. The levels and characteristics of these environmental factors were related to the various human activities taking place around the mosquito breeding sites. Analyses of the significance of these findings and observations and inferences from previous studies have demonstrated the impact this study could produce on the contemporary integrated vector control approach to malaria management.

CHAPTER SIX

6.0 Relationship between Glutathione Levels and Activities of Detoxification Enzymes in *Anopheles gambiae*

6.1 Introduction to Glutathione

Aerobic organisms during metabolic processes produce reactive oxygen species as by-products. Most of these reactive species are toxic to the organism and have to be eliminated from the system. To do this, the organisms synthesize intracellular thiols such as glutathione (GSH), homoglutathione, γ-glutamyl-cysteine (γ-Glu-CYS), γ-glutamyl-cystenylserine and mycothiol (Carnegie, 1963; Newton and Javar, 1985; Klapheck *et al.*, 1992; and Newton *et al.*, 1996). Of all these thiol molecules, glutathione is the most important. In addition to serving as substrate for glutathione S- transferase which detoxify potentially dangerous electrophiles, GSH also provides reducing equivalents to several enzymes including ribonucleotide reductase, 3'-phosphoadesonosyl5'-phosphosulfate reductase and arsenate reductase (Russell *et al.*, 1990; Gladysheia *et at.*, 1994). It also serves as a buffer to maintain the redox status of the cytoplasm and protect biomolecules against oxidative damage (Copley and Dhillon, 2002). Glutathione is found in virtually all eukaryotes and some prokaryotes. In prokaryotes, it is found mainly in Gram-negative bacteria and rarely in Gram-positive. GSH is not present in Archea or amitochondrial eukaryotes such as *Entamoeba hystolica, Giardia duodenalis, Trichomonas vaginalis,* and *Trichomonas foetus* (Brown *et al.*, 1993; Ellis *et al.*, 1994). This distinctive pattern of distribution has lead to suggestion that eukaryotic GSH may have been inherited from bacteria via the projenitor of mitochondria (Fahey, 2001; Yang *et al.*, 2008). If this suggestion is true, then the GSH biosynthetic genes in eukaryotes should share similarities with those of alpha-proteobacteria, the modern relatives of the mitochondrial projenitor (Gray, 1993). GSH is synthesized in two separate ATP-driven enzyme catalysed reactions. The two major enzymes involved are γ-Glutamylcysteine ligase

(GCLC) which catalyses the formation of a peptide bond between γ-carboxylate of glutamate and cysteine and GSH synthetase (GS) which catalyses the formation of a second peptide bond between the cysteinyl carboxylate of γ-Glu-Cys and the amino acid group of glycine (Copley and Dhillon, 2002).

The active group of glutathione is the SH-group of its cysteine residue. Although present in all organs, GSH is much greater in the liver due to its role in drug and xenobiotic metabolism. GSH is present either in its reduced (GSH) or oxidized (GSSG) forms. It can also be bound to many proteins to form glutathionylated proteins. Under normal condition, GSH is present mostly in the reduced form but converted to the oxidized form during oxidative stress. The oxidized form can be converted back to the reduced form through the action of the enzyme glutathione reductase. Thus, the ratio of the reduced to oxidized form of glutathione indicate the redox state of the cell. The γ-glutamyl linkage and the presence of sulfhydryl group in GSH allow it to participate in a number of physiological activities. By participating in transhydrogenation reactions, GSH serves to maintain the sulfhydryl groups of other protein and non protein thiols such as coenzyme A. The presence of sulfhydryl group also allows glutathione to provide reducing capacity for several reactions including formation of deoxyribonucleotides by ribonucleotide reductase, and the reduction of dehydroascorbate to ascorbate. Another major function of glutathione is the metabolism of hydrogen peroxide, other peroxides, free radicals as well as various environmental and indigenous xenobiotics (Pastore *et al.*, 2003). Because of these and many other functions of glutathione, monitoring the intracellular levels of its various forms is very important in understanding how GSH homeostatis could be affected under various conditions.

The aim of this chapter therefore, is to investigate the differential levels of different forms of glutathione and to establish correlations between these levels and the activities of detoxification enzymes in the three life stages of *An. gambiae* sampled from three different

breeding ecologies in Nigeria (Table 3.1). This will provide information on the differential levels of the three major forms of glutathione (total, oxidized and reduced) in *An.* mosquitoes thriving in different breeding ecologies in Nigeria. Then, association between levels of the different forms of GSH and activities of the three major detoxification enzymes (GST, P450, α and β-esterases) will also be examined in order to evaluate their relationships with these enzymes in the overall detoxification process in *An. gambiae*. This is relevant in view of the role of glutathione in both enzymatic and non enzymatic xenobiotic detoxification. Findings from this study could allow for an evaluation of the possibility of targeting control of the activities of detoxification enzymes in mosquitoes through manipulation of the availability of GSH.

6.2 Results

6.2.1 Assessing the concentration or Levels of the Different forms of Glutathione across the three Life Stages of *Anopheles gambiae*

In order to investigate the relationship and correlation between levels of the three forms of glutathione (total GSH, GSG and GSSG) and activities of these detoxification enzymes in *An. gambiae*, an assay of total glutathione (tGSH) and oxidized glutathione (GSSG) was carried out across the three life stages of *An. gambiae* mosquitoes. The *Anopheles* mosquito samples were collected from three different breeding ecologies (Table 3.1) in Nigeria. The procedure employed for the assay is based on the enzyme recycling method described by Teitze (1969). The general thiol reagent, 5',5'-dithiobis[2-nitrobenzoic acid] (DTNB, Ellman's Reagent) reacts with GSH to form 5-thionitrobenzoic acid (TNB) which can be measured photometrically at 412nm. In order to measure the levels of total glutathione (tGSH), any oxidized glutathione (GSSG) present in the sample was converted to GSH by glutathione reductase. Levels of oxidized glutathione was determined by first removing or quenching all

reduced GSH present with 4-vinyl pyridine before addition of the Ellman's reagent and glutathione reductase. The difference between total and oxidized glutathione was used to obtain the levels of reduced GSH. All the glutathione levels were corrected for the milligrams of protein present in the samples and expressed as nmol/mg protein. (See Chapter two for Methodology).

Table 6.1 Total Glutathione (tGSH), Reduced glutathione (GSH), and Oxidized Glutathione (GSSG) levels (nmol/mg protein) in the larval stage of *An. gambiae* sampled from three different breeding ecologies in Nigeria.

Sites	Total GSH[a]	GSH	GSSG	GSH: tGSH[b]	GSSG: GSH[c]	GSSG: tGSH[d]	Study Zone[e]
1	83.57±2.41	71.87±2.08	11.70±0.33	0.86	0.16	0.14	
2	59.36±2.58	51.43±2.21	8.31±0.36	0.87	0.16	0.13	A
3	63.97±3.59	55.01±3.09	8.95±0.51	0.86	0.16	0.14	
1	81.60±3.62	73.61±3.27	7.98±0.34	0.90	0.11	0.10	
2	74.10±2.00	66.84±1.80	7.23±0.15	0.90	0.11	0.10	B
3	57.87±2.40	52.19±2.17	5.67±0.23	0.90	0.11	0.10	
4	61.10±4.55	55.11±4.10	5.98±0.45	0.90	0.11	0.10	
1	62.37±2.54	52.39±2.14	9.97±0.40	0.84	0.19	0.16	
2	51.80±4.05	43.51±3.40	8.29±0.65	0.84	0.19	0.16	C
3	70.77±2.95	59.44±2.48	11.32±0.47	0.84	0.19	0.16	

[a]Mean ± S.D for three determinations.

[b, c, d]Ratios of reduced to total; oxidized to reduced; and oxidized to total glutathione respectively.

[e]Zone: A, intensive agriculture; B, residential/domestic; and C, petrochemical breeding ecologies

Table 6.2 Total Glutathione (tGSH), reduced glutathione (GSH), and Oxidized Glutathione (GSSG) levels (nmol/mg protein) in the Pupal stage of *An. gambiae* sampled from three different breeding ecologies in Nigeria.

Sites	Total GSH[a]	GSH	GSSG	GSH: tGSH[b]	GSSG: GSH[c]	GSSG: tGSH[d]	Study Zone[e]
1	61.63±3.11	48.08±2.42	13.56±0.68	0.78	0.28	0.22	
2	61.77±1.39	48.18±1.10	13.59±0.31	0.78	0.28	0.22	A
3	48.77±3.25	38.04±2.54	10.73±0.72	0.78	0.28	0.22	
1	66.80±1.28	57.45±1.10	9.35±0.18	0.86	0.16	0.14	
2	58.00±2.41	49.88±2.07	8.12±0.34	0.86	0.16	0.14	B
3	50.77±3.67	43.66±3.16	7.11±0.51	0.86	0.16	0.14	
4	60.83±2.35	52.32±2.02	8.52±0.33	0.86	0.16	0.14	
1	46.50±3.40	39.06±2.85	7.44±0.54	0.84	0.19	0.16	
2	42.27±2.64	35.50±2.22	6.76±0.42	0.84	0.19	0.16	C
3	58.43±2.57	49.08±2.16	9.35±0.41	0.84	0.19	0.16	

[a]Mean ± S.D for three determinations.

[b, c, d]Ratios of reduced to total; oxidized to reduced; and oxidized to total glutathione respectively.

[e]Zone: A, intensive agriculture; B, residential/domestic; and C, petrochemical breeding ecologies

Table 6.3 Total Glutathione (tGSH), reduced glutathione (GSH), and Oxidized Glutathione (GSSG) levels (nmol/mg protein) in the Adult stage of *An. gambiae* sampled from three different breeding ecologies in Nigeria.

Sites	Total GSH[a]	GSH	GSSG	GSH: tGSH[b]	GSSG: GSH[c]	GSSG: tGSH[d]	Study Zone[e]
1	53.77±3.10	39.18±2.95	14.57±0.38	0.73	0.37	0.27	
2	47.77±3.17	34.80±2.94	12.96±0.49	0.73	0.37	0.27	A
3	49.60±0.98	36.12±0.89	13.48±0.86	0.73	0.37	0.27	
1	49.93±2.42	43.91±2.22	6.02±0.23	0.88	0.14	0.12	
2	48.77±0.95	42.87±0.71	5.90±0.24	0.88	0.14	0.12	B
3	47.40±1.40	41.67±1.32	5.73±0.11	0.88	0.14	0.12	
4	47.73±1.10	41.98±1.00	5.76±0.14	0.88	0.14	0.12	
1	41.97±0.75	34.44±0.42	7.52±0.84	0.82	0.22	0.18	
2	32.77±3.78	26.89±3.00	5.87±0.97	0.82	0.22	0.18	C
3	48.63±2.21	39.91±1.05	8.73±1.22	0.82	0.22	0.18	

[a]Mean ± S.D for three determinations.

[b, c, d]Ratios of reduced to total; oxidized to reduced; and oxidized to total glutathione respectively.

[e]Zone: A, intensive agriculture; B, residential/domestic; and C, petrochemical breeding ecologies

Data in Tables 6.1-6.3 showed the distribution of the three forms of glutathione (total, oxidized and reduced) across the three life stages of *An. gambiae* sampled from three different breeding ecologies in Nigeria. Also, the ratios of the oxidized and reduced, relative to the total glutathione, were described. According to these ratios, more than 70% of glutathione is present in the reduced form across the three life stages and across the three study zones.

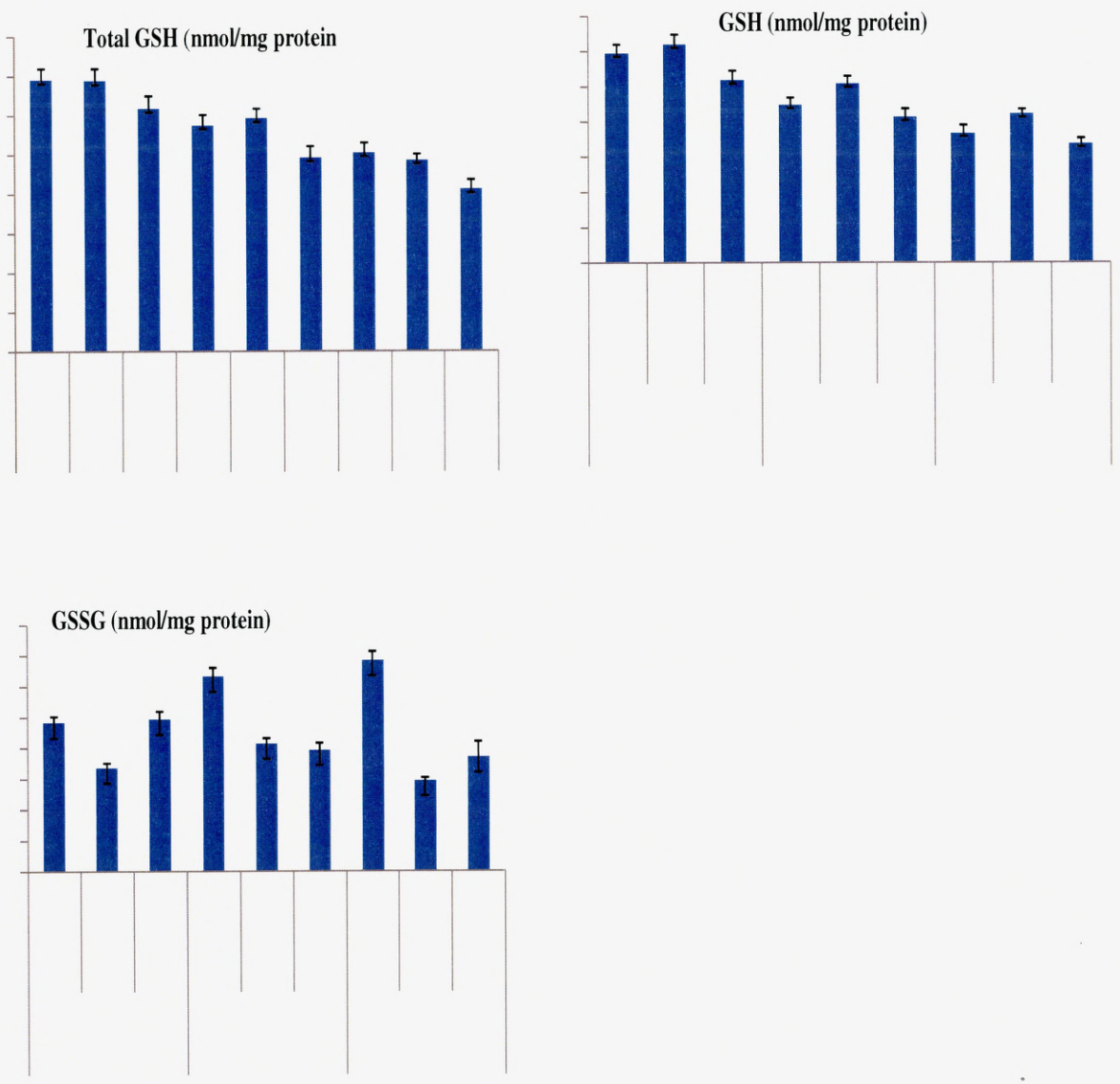

Fig. 6.1 Distribution of total, reduced and oxidized glutathione across the three life stages (Larvae, Pupae and Adult) of *An. gambiae*. The *Anopheles* mosquitoes were collected from three different breeding ecologies, designated as study zones, in Nigeria. Zone A; intensive agriculture, B; domestic/residential areas and C; petrochemical/hydrocarbon laden breeding sites. Concentration of glutathione was determined as described in Materials and Method section.

The relative distribution of glutathione (Figure 6.1) showed that the levels of total and reduced GSH across the three study zones appeared to be similar while the levels of oxidized glutathione (GSSG) appeared to be higher in study zone A and C (intensive agriculture and petrochemical laden respectively) compared to zone B (domestic/residential areas). GSSG in

zone A was about 1.75 fold higher than those of zone B, especially in the pupal and adult life stages. GSSG in zone C was about 1.5-fold higher than that of zone B in the larval stage; the levels between these two zone appeared to be similar in the pupal stage, while in the adult stage, zone C was about 1.3-fold higher than B. Furthermore, total and reduced glutathione concentration appeared to be higher in the larval stages compared to the two other life stages (Fig. 6.1). A graphical comparism of the distribution of these three forms of GSH across the three study zones is presented in Figures 6.2, 6.4 & 6.6.

6.2.2 Relationship between Levels of the three forms of Glutathione and Activities of Detoxification Enzymes

In order to evaluate the role of glutathione in xenobiotic detoxification and the impact of its differential levels on the activities of detoxification enzymes in *An. gambiae*, the relationship and correlations between levels of total, oxidized and reduced forms of glutathione and the activities of detoxification enzymes were examined across the three life stages of *An. gambiae*. Similar statistical tools in SPSS v.20, employed in chapters 4 and 5, were also applied here to investigate these relationships. The results and observations recorded are presented below:

6.2.2.1 Mean Distribution of the three forms of Glutathione and Association between these forms and the Activities of Detoxification Enzymes.

a). Total Glutathione (tGSH)

The result of the one way ANOVA, which was used to investigate the differential mean distribution of total glutathione (tGSH) across the three study zones, showed that there were no significant differences ($p= 0.562$ and 0.139) in mean distribution of total glutathione at the larval and pupal stages of *An. gambiae* respectively. The mean distribution at the adult stage

was moderately significant (p= 0.033). While the significance difference in distribution was low in the two aquatic immature stages (larvae and pupae) and moderate in the adult stage, the p-values nonetheless showed a gradual progression in significance across the three life stages i.e. from larvae to adult (Fig. 6.2).

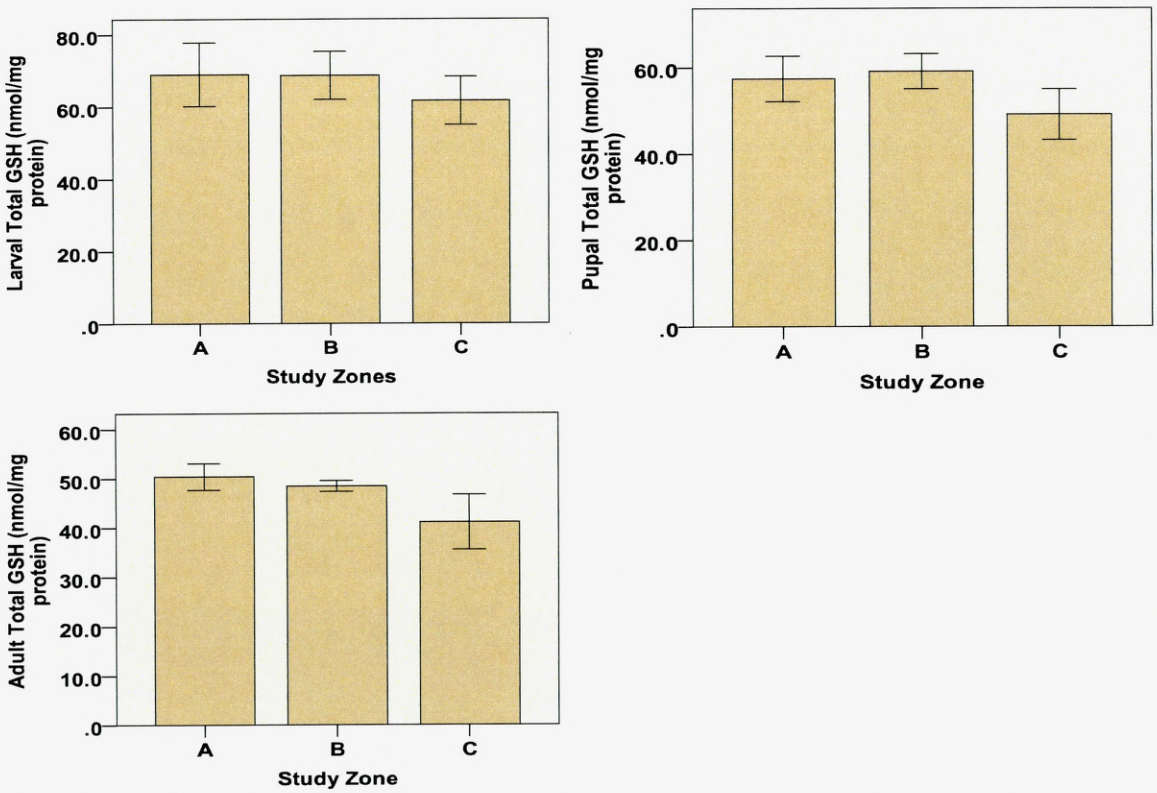

Fig. 6.2 Mean distribution of total glutathione (tGSH) across the three life stages; Larvae, Pupae and Adults, of *An. gambiae*. The *An. gambiae* mosquitoes were sampled from three different breeding ecologies categorized into three study zones in Nigeria. Zone A; Intensive agriculture; B; Domestic/residential areas and C; petrochemical/hydrocarbon laden breeding ecologies. Total glutathione levels were determined as described in Materials and Method Section.

Bonferoni pairwise comparism test was used to examine zone-wise differences in mean total glutathione distribution across the three study zones. The result showed that at the larval stage, no significant difference (p=1.000) was detected when the three zones were compared against one another. The degree of significance improved a little at the pupal stage between A and C and B and C (p= 0.394 and 0.181) respectively while that between A and B remain the same (p=1.000). Finally, further increase in the degree of significance was recorded at the

adult stage between A & C and B & C (p= 0.045 and 0.097) respectively. The pairwise significance between A and B remain the same at this stage (p=1.000) as in the two lower life stages (larvae and pupae). This means that the result of Bonferoni pairwise comparism followed the same pattern as that of the Mixed Linear Model; significance increases across the life stages from the lowest to the highest (i.e. from larvae to adult).

Furthermore, the result of the Linear Regression model, which examined the correlation or association between levels of total glutathione and activities of P450, GST and α/β-esterase, showed that there was a moderate significant association with P450, which increases across the life stages, while there was little or no association between total glutathione and GST and α/β-esterase activities (Fig. 6.3). Total glutathione was moderately negatively associated (p= 0.319, 0.051 and 0.026) with P450 activities at the larval, pupal and adult life stages respectively. However, the association or correlation between total glutathione and GST and α/β-esterase were not highly significant. The p-values for GST at the larval, pupal and adult life stages were 0.987, 0.707 and 0.089 respectively. The p-values for α-esterase at the larval, pupal and adult life stages were 0.505, 0.794 and 0.014 respectively while p-values for β-esterase at the larval, pupal and adult stages were 0.886, 0.476 and 0.060 respectively. While the association was not highly significant for GST and α/β-esterases and moderately significant for P450, the significance for the association between total glutathione and the three detoxification enzymes (Fig. 6.3), nonetheless improved across the three life stages (See Appendix for Larval and Pupal Correlation Figures) .

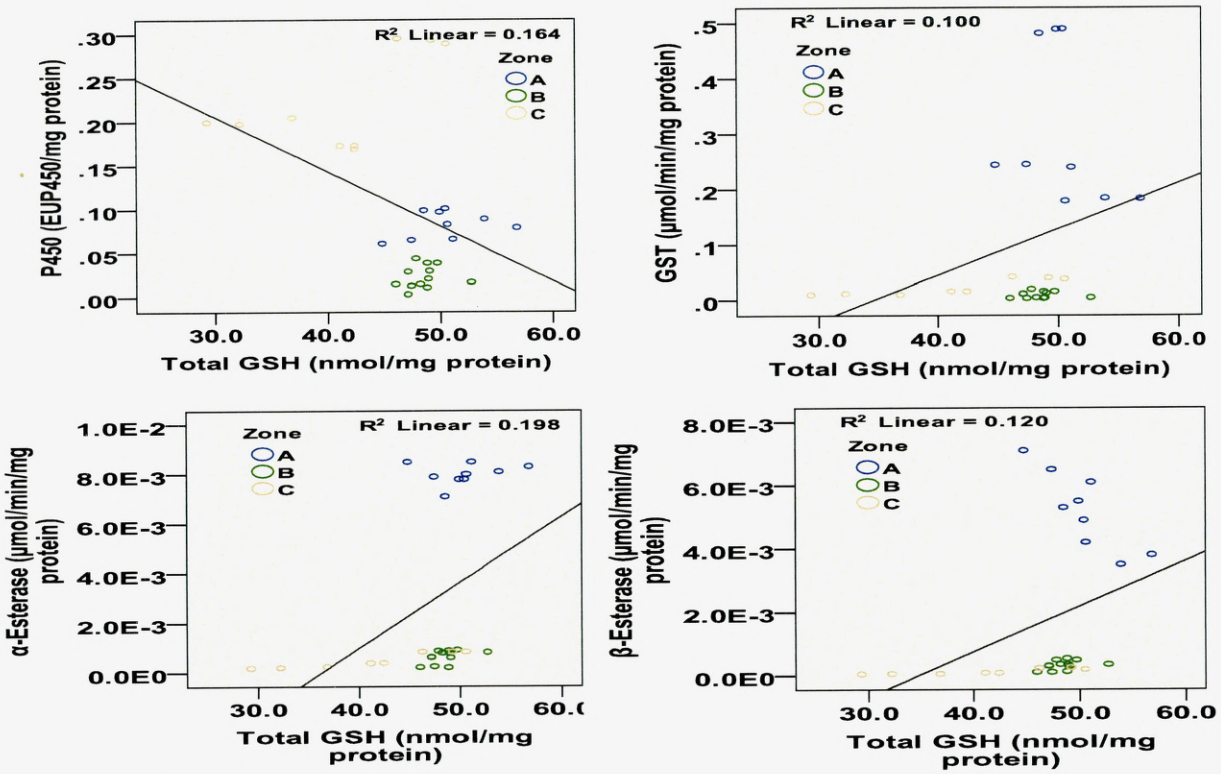

Fig. 6.3 Associations/correlations between total glutathione (tGSH) and activities of P450, GST and α & β-esterases at the adult stage of *An. gambiae*. The *An. gambiae* mosquitoes were sampled from three different breeding ecologies categorized into three study zones in Nigeria. Zone A; Intensive agriculture; B; Domestic/residential areas and C; petrochemical/hydrocarbon laden breeding ecologies. Total glutathione levels and the activities of the enzymes were determined as described in Materials and Method Section.

b). Reduced Glutathione (GSH)

The result of one way ANOVA which was used to evaluate the differential mean distribution of reduced glutathione (GSH) across the three study zones showed some level of similarities with those recorded for total glutathione (Fig. 6.2) No significant difference (p= 0.300 and 0.088) was observed in mean GSH distribution at the larval and pupal stages of *An. gambiae* respectively. However, as in total GSH, the adult life stage recorded a moderate significant difference (p= 0.011) in mean distribution of GSH across the three study zones (Figure 6.4).

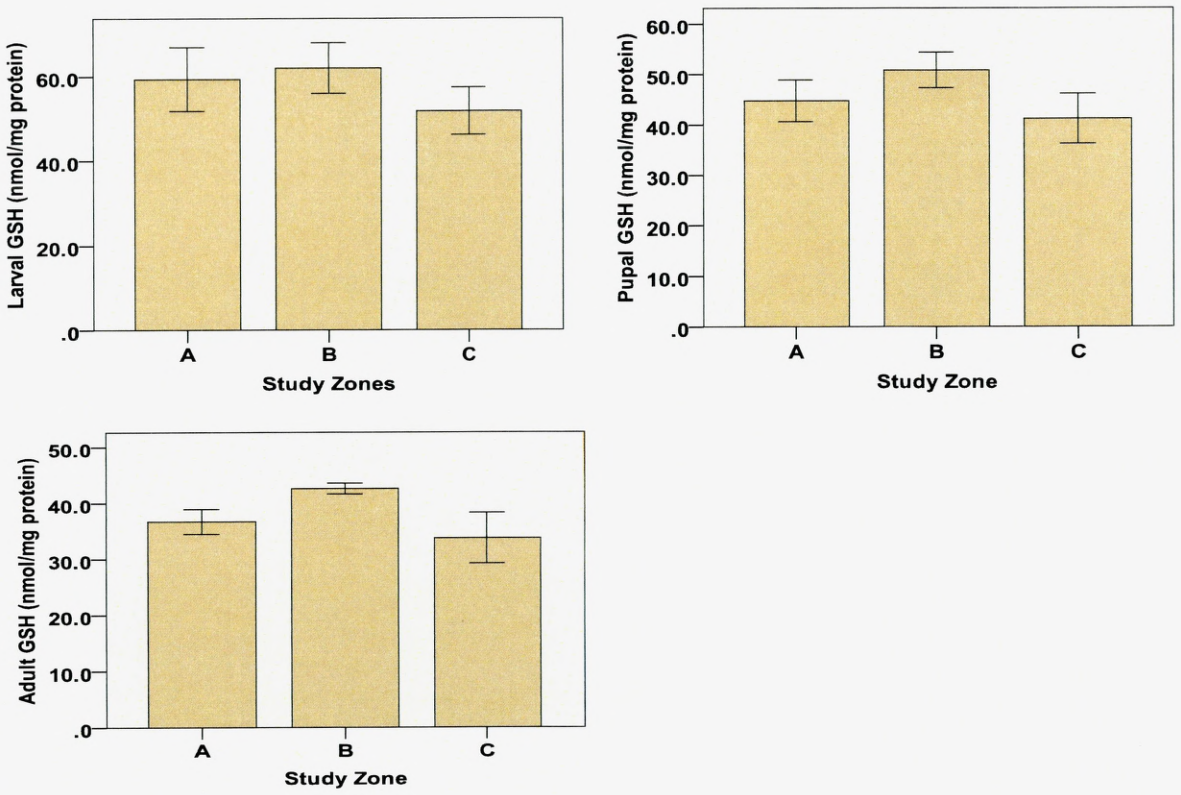

Fig. 6.4 Mean distribution of reduced glutathione (GSH) across the three life stages; Larvae, Pupae and Adults, of *An. gambiae*. The *An. gambiae* mosquitoes were sampled from three different breeding ecologies categorized into three study zones in Nigeria. Zone A; Intensive agriculture; B; Domestic/residential areas and C; petrochemical/hydrocarbon laden breeding ecologies. Total glutathione levels were determined as described in Materials and Method Section.

In contrast to total glutathione, the result of Bonferoni pairwise comparism showed that different p-values were recorded for each zone-wise comparism across the three life stages. At the larval stage, there was no significance differences (p= 1.000, 0.862 and 0.408) for zone-wise mean GSH comparism between zone A against B; A against C; and B against C respectively. There were also little or no significant differences (p= 0.463, 1.000 and 0.104) when zone A & B; A & C and B & C respectively, were compared. Finally, as observed in total glutathione, the significance improved a little at the adult stage Bonferoni pairwise comparism of mean reduced glutathione across the three study zones. The mean distribution were however also not highly significant (p=0.101, 0.828 and 0.012) for zone A against B; A against C and B against C zone-wise comparisms respectively.

The results of the Linear Regression analysis for reduced glutathione were also very similar to those of total glutathione. Reduced glutathione was significantly negatively associated (0.058, 0.010 and 0.004) with larval, pupal and adult stage P450 activities respectively. This means that as the P450 activities increases, the levels of reduced glutathione decreases. Also, the strength of this association increases across the three life stages i. e. from larvae to adult life stage. In contrast however, there were no significant association between levels of reduced glutathione and the activities of GST and α/β-esterases (Fig. 6.5). For GST, the p-values at the larval, pupal and adult life stages were 0.787, 0.112 and 0.283 respectively. For α-esterase, the p-values at the larval, pupal and adult stages were 0.721, 0.283 and 0.448 respectively while for β-esterase, the p-values recorded at the larval, pupal and adult stages were 0.921, 0.493 and 0.273 respectively. While not highly significant, the p-values for the association between reduced glutathione and GST and α/β-esterases also indicate that the degree or strength of the association increases across the life stages. (See Appendix for Larval and Pupal Correlation Figures).

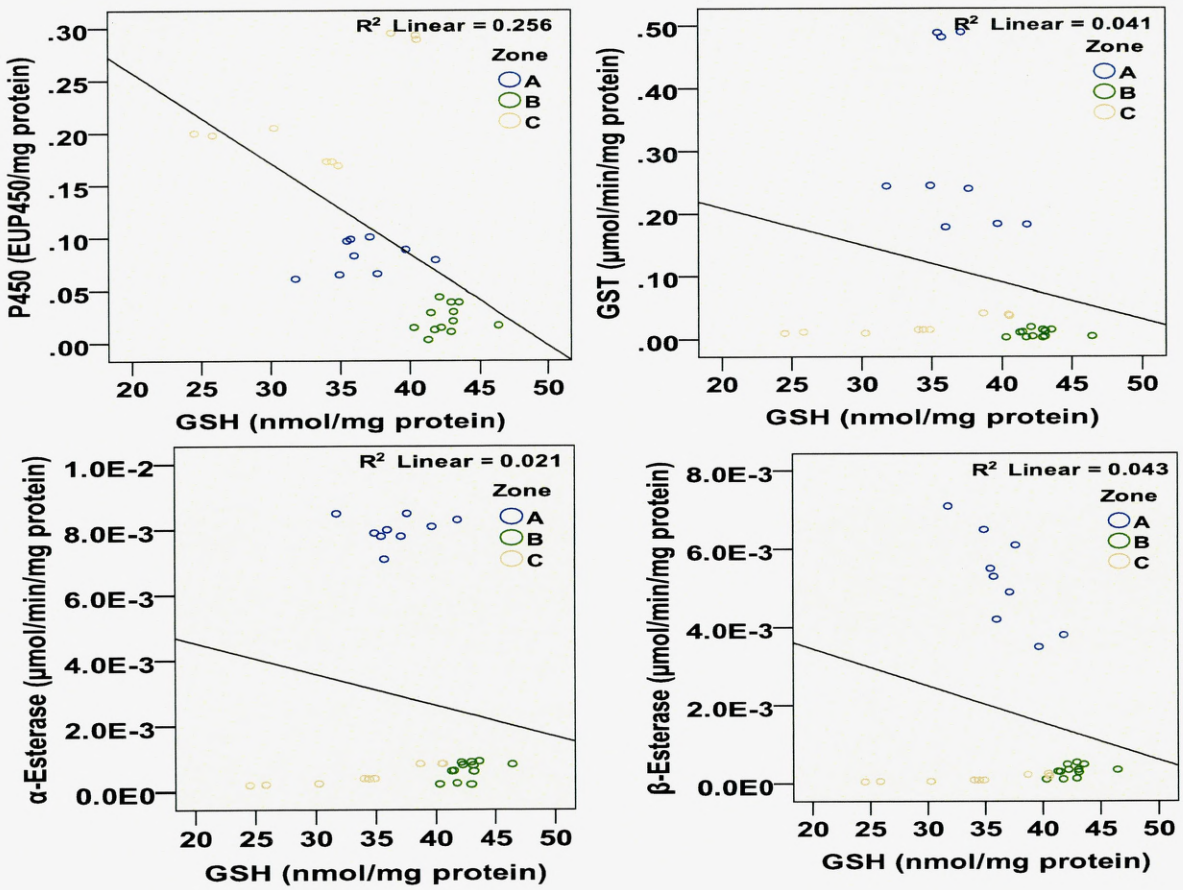

Fig. 6.5 Associarion/correlation between reduced glutathione (GSG) and activities of P450, GST and α & β-esterases at the adult stage of *An. gambiae*. The *An. gambiae* mosquitoes were sampled from three different breeding ecologies categorized into three study zones in Nigeria. Zone A; Intensive agriculture; B; Domestic/residential areas and C; petrochemical/hydrocarbon laden breeding ecologies. Total glutathione levels and the activities of the enzymes were determined as described in Materials and Method Section.

c). Oxidized Glutathione (GSSG)

The results of the evaluation of the relationship between levels of oxidized glutathione (GSSG) and the activities of the detoxification enzymes was markedly different from those of the other two GSH forms (tGSH and GSH). Firstly, the result of the one way ANOVA, which was used to analyse the differential mean distribution of GSSG across the three study zones, showed that there were statistically significant differences (p= 0.010, 0.000 and 0.000) in distribution of GSSG at the larval, pupal and adult life stages respectively, across the three

study zones (Fig. 6.6). As in the other two forms of glutathione described above, the degree of significance also appeared to increase across the three life stages.

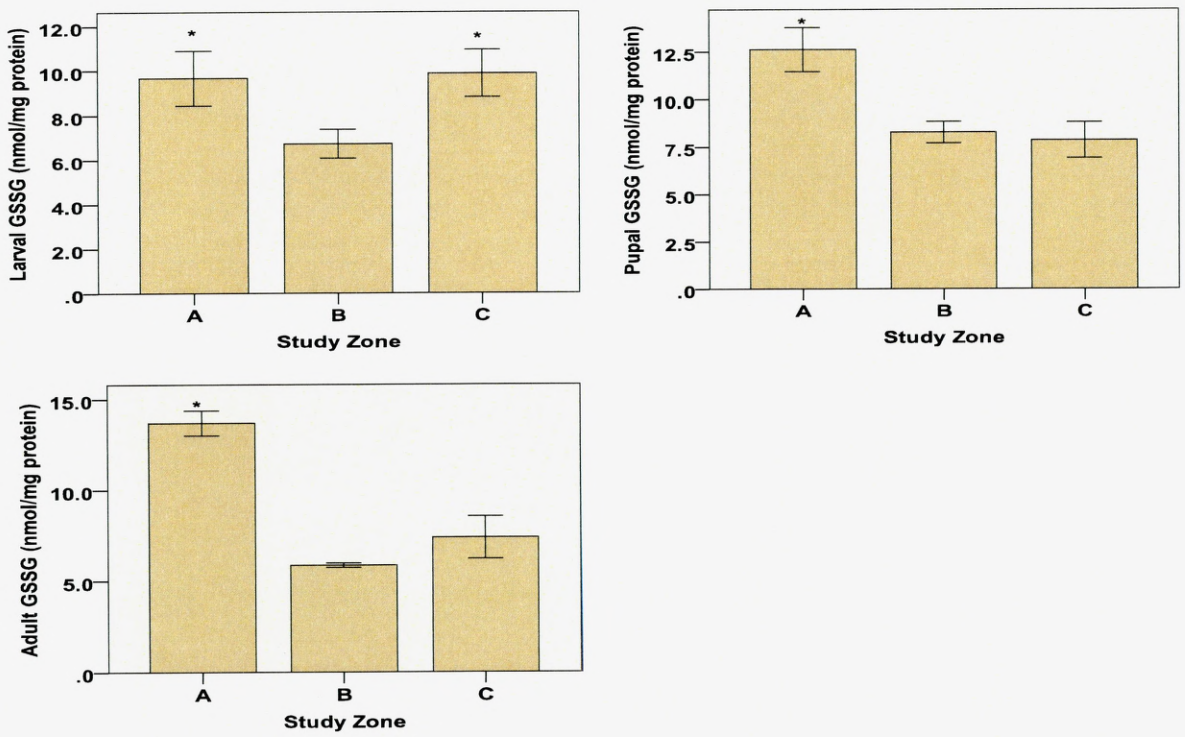

Fig. 6.6 Mean distribution of oxidized glutathione (GSSG) across the three life stages; Larvae, Pupae and Adults, of *An. gambiae*. The *An. gambiae* mosquitoes were sampled from three different breeding ecologies categorized into three study zones in Nigeria. Zone A; Intensive agriculture; B; Domestic/residential areas and C; petrochemical or hydrocarbon laden breeding ecologies. Total glutathione levels were determined as described in Materials and Method Section. * indicates significant values ($p<0.05$) relatives to those of the other zones.

The result of Bonferoni pairwise comparism showed that zone A & B and B & C pairwise comparism were highly significant (p= 0.030 and 0.021) respectively, while that between A and C was not statistically significant (p= 1.000) at the larval stage of *Anopheles gambiae*. However, at the pupal stage, comparism between A & B and A & C was statistically significant (p= 0.001) while that between B & C was not (p= 1.000). Finally, as observed with the two other forms of glutathione, the degree of significance increased at the adult life stage. A & B and A & C zone-wise comparisms recorded p-values of 0.000 while B against C recorded p-values of 0.069.

Furthermore, result of Linear Regression Model established associations between oxidized glutathione and the activities of the three major detoxification enzymes (i.e. P450, GST and α & β-esterases). There was a statistically significant positive association or correlation (p= 0.000) between GSSG and larval P450 activities, although significance (p= 0.449 and 0.663) decreased at the pupal and adult stages respectively. In contrast however, GST and α & β-esterases showed highly statistically positive associations (p= 0.000) with levels of GSSG across all the three life stages (Fig. 6.7). The p-values for association between GSSG and GST activities at the larval, pupal, and adult stages of *An. gambiae* were 0.151, 0.000 and 0.000 respectively. For α-esterase activities, the p-values at the larval, pupal, and adult stages were 0.070, 0.000 and 0.000 respectively while associations between GSSG and larval, pupal and adult stage β-esterase activities recorded p-values of 0.205, 0.000 and 0.000 respectively. These results were in line with the previous observations made with the other two forms of glutathione (Section 6.3.2 a and b): significance in distributions and associations appeared to increase across the three life stages (See Appendix for Larval and Pupal stage Correlation Figures).

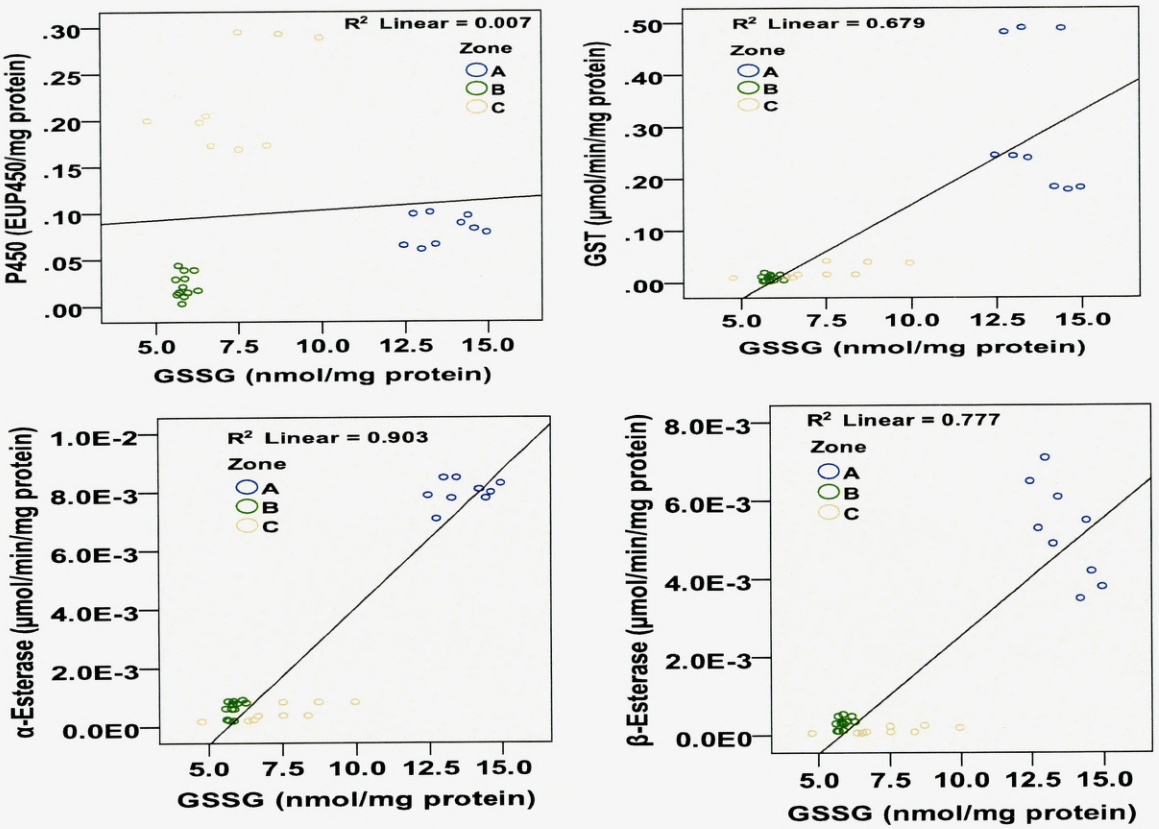

Fig. 6.7 Association/correlation between oxidized glutathione (GSSG) and activities of P450, GST and α & β-esterases at the adult stage of *An. gambiae*. The *An. gambiae* mosquitoes were sampled from three different breeding ecologies categorized into three study zones in Nigeria. Zone A; Intensive agriculture; B; Domestic/residential areas and C; petrochemical/hydrocarbon laden breeding ecologies. Total glutathione levels and the activities of the enzymes were determined as described in Materials and Method Section.

6.2.2.2 Effect of the three forms of Glutathione on Activities of the Detoxification Enzymes.

Due to the results of the factor analysis conducted on the detoxification enzyme variables (Chapter 5; Section 5.2.2.2) which extracted the three enzymes as principal components irrespective of the *An. gambiae* life stage, it was necessary to perform the same analysis on the three forms of glutathione (total, oxidized and reduced) studied, so as to also extract the glutathione principal components that explains all the variability in these forms of glutathione across the three life stages studied. Then Redundancy analysis could also be used to

determine the glutathione components that produce a combined effect on the extracted principal components of the detoxification enzymes activities.

The results of the factor analysis on the three forms of glutathione studied across the three life stages of *An. gambiae* showed that five principal components (PC) (See Appendix for both Total Variance and Rotated Component Matrix) were extracted and they explained more 99% of the variability in the overall glutathione variables. PC 1 correlates strongly with total and reduced GSH at pupal stage, PC 2 was strongly associated with only oxidized (GSSG) glutathione at both pupal and adult stages, PC 3 correlates strongly with total and reduced GSH at larval stage, PC 4 was explained by total and reduced glutathione at the adult stage and finally PC 5 correlates strongly with only GSSG at the larval stage of *An. gambiae*. Thus in summary, PCs 1, 3, and 4 correlated strongly with both total and reduced glutathione (tGSH & GSH) across the three life stages while PCs 2 and 5 was strongly associated with oxidized glutathione (GSSG) also across the three life stages. Thus in contrast to the results of factor analysis for the detoxification enzyme variables (Chapter 5, Section 5.2.2.2), the life stages of *An. gambiae* appeared to produce an impact on the variability of the three forms of glutathione studied. This results was consistent with the results of the Linear Regression Analysis (Section 6.2.2.1) carried out between the levels of these three forms of glutathione and the detoxification enzyme activities.

Data in the diagram below (Fig. 6.8) showed the five glutathione extracted components that explained more than 99% of the variability among the nine glutathione variables.

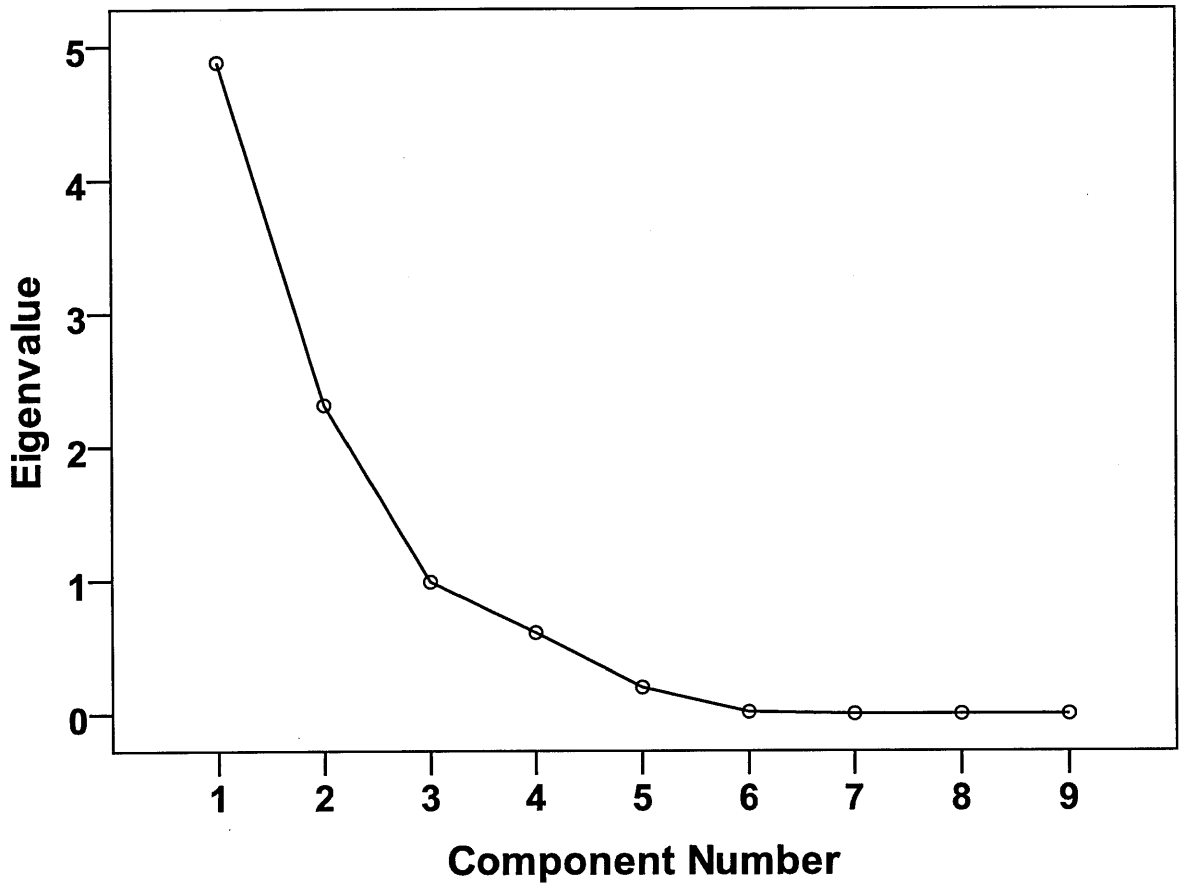

Fig. 6.8. Scree Plot of the extracted principal components from the factor analysis of the glutathione variables. PCs 1-5 explained 99% of the variability in the data.

Finally, Redundancy analysis was carried between the extracted PCs of the glutathione variables and those of the detoxification enzymes to determine the combination of the forms of glutathione that produce a combined effect on each of the three detoxification enzyme factors. The result of the redundancy analysis (Table 6.4) between the glutathione extracted PCs and GST and α & β-esterase showed that a combination of total and reduced glutathione (tGSH & GSH) at only the pupal stage and oxidized glutathione at all the three stages (PCs 1, 2, and 5) produced a combined effect on GST and α & β-esterase activities.

Table 6.4 Glutathione Factors or Components with Combined Effect on GST and α & β-esterases across the three Life Stages of *An. gambiae* .

			95% Wald Confidence Interval		Hypothesis Test		
Parameter	**B**	**Std. Error**	**Lower**	**Upper**	**Wald Chi-square**	**df**	**Sig.**
Intercept	-1.032E-015	0.0405	-0.079	0.079	0.000	1	1.000
PC 1[a]	-0.114	0.0412	-0.194	-0.033	7.586	1	0.006
PC 2	0.965	0.0412	0.884	1.045	547.384	1	0.000
PC 3	0.009	0.0412	-0.072	0.090	0.051	1	0.822
PC 4	0.012	0.0412	-0.069	0.093	0.085	1	0.770
PC 5	-0.074	0.0412	-0.154	0.007	3.191	1	0.074
Scale	0.049[b]	0.0127	0.030	0.082			

[a]: Refers to the etracted principal components from the glutathione variables.

[b]:Maximum likelihood estimate

Lastly, the result of the redundancy analysis (Table 6.5) between the glutathione extracted PCs and P450 activities showed that four out of the five glutathione extracted principal components (PCs 1, 3, 4, and 5) produced a combined effect on P450 activities. Thus total and reduced glutathione at the three life stages (PCs 1, 3, and 4), and oxidized glutathione at only the larval stage were responsible for the combined effect of glutathione on the activities of P450 in *An. gambiae* from Northern Nigeria.

Table 6.5 Glutathione Factors or Components with Combined Effect on P450 Activities across the three Life Stages of *Anopheles gambiae* .

			95% Wald Confidence Interval		Hypothesis Test		
Parameter	**B**	**Std. Error**	**Lower**	**Upper**	**Wald Chi-square**	**df**	**Sig.**
Intercept	8.957E-016	0.0505	-0.099	0.099	0.000	1	1.000
PC 1[a]	-0.236	0.0513	-0.337	-0.136	21.218	1	0.000
PC 2	0.012	0.0513	-0.089	0.133	0.055	1	0.815
PC 3	-0.304	0.0513	-0.404	-0.203	35.029	1	0.000
PC 4	-0.296	0.0513	-0.396	-0.195	33.254	1	0.000
PC 5	0.828	0.0513	0.727	0.928	260.239	1	0.000
Scale	0.076[a]	0.0197	0.046	0.127			

[a]: Refers to the etracted principal components from the glutathione variables.

[b]:Maximum likelihood estimate

6.3 Discussion

This study demonstrated the presence of the two forms of glutathione; oxidized and reduced, their role in xenobiotic detoxification process and their relationship with detoxification enzymes across the three life stages of *An. gambiae*. Glutathione plays a vital role in both enzymatic and non enzymatic routes for xenobiotic detoxification in most organisms. Similar process of regulatory mechanisms has been reported for both glutathione and many of the detoxification systems in organisms (Matsuiki *et al.*, 2008; Pastore *et al.*, 2003). Furthermore, availability and levels of the different forms of glutathione, as well as synthesis and activities of detoxification enzymes have been found to responds to changes in the degree of oxidative stress induced by xenobiotic overload in different organisms (Lin and Yang, 2007; Xu *et al.*, 2005). The ubiquitous nature of mosquito breeding ecologies in most malaria endemic

countries means that mosquito breeding sites can be created in varieties of environments where they could be exposed to arrays of different environmental xenobiotics resulting from various human related activities (Budiansky, 2002; Strode *et al.*, 2006). Due to these observations, monitoring the levels of various forms of glutathione and examining the relationship between these forms and activities and the major detoxification enzymes in *An. gambiae* is necessary to provide an alternative approach for management of insecticides resistance, hence this study.

The *An. gambiae* in this study were sampled from three different breeding ecologies defined by the type of human related activities taking place within and/or around the mosquito breeding sites (Table 3.1). Levels of physical environmental factors (i.e. pH. Temperature, conductivity, transparency, dissolved oxygen and biological oxygen demand) and chemical environmental parameters (i.e. total dissolved solids, sulphates, phosphates, nitrites, nitrates, carbon content and oil and gas) were determined from all the *An. gambiae* breeding sites visited across the three studied zones. The results (Chapter 4, Section 4.2) showed that the distribution of the levels of many of these factors (especially the chemical environmental parameters) varied significantly across the three studied zones. Furthermore, activities of the three major detoxification enzymes (GST, P450 and α & β-esterases) were determined in the three life stages of *An. gambiae* collected from all the breeding sites across the zones. The results (Chapter 5, Section 5.2) also showed that the mean activity distribution of the three enzymes varied significantly across the three study zones.

In this present study, levels per mg protein, of three forms of glutathione; total, oxidized and reduced, were determined in the three life stages of *An. gambiae* collected from breeding sites located across the three study zones. The relative zone-wise mean distribution of these forms of glutathione and the correlations or associations between their levels and the activities of the three detoxification enzymes (P450, GST and α & β-esterases) was investigated. The

results showed that the three forms of glutathione were all detected in the three life stages of *An. gambiae* in varying concentration across the three studied zones. Over 70% of the assayed glutathione was present in the reduced forms (GSH) across the three life stages. Also, the levels of total tGSH) and reduced (GSH) glutathione appeared to be higher in the larval stage of *An. gambiae* compared to the other two stages. There was not much variation between the pupal and adult stages (Fig. 6.1). These observations are consistent with the findings of a previous study (Hazelton and Lang, 1984) which investigated the relationship between ageing and glutathione levels in mosquito.

Furthermore, there was a low to moderate significant differences in the mean distribution of total and reduced glutathione levels across the three study zones which appeared to increase across the life stages (Fig. 6.2, and 6.4). However, the mean distribution of oxidized glutathione (GSSG) across the three zones was highly statistically significant ($p<0.05$), with higher concentrations recorded in study zone A and C compared to B. Similar patterns of distribution was recorded for the environmental chemical factors (Chapter 4) and the activities of the detoxification enzymes (Chapter 5). In addition, results of the Linear Regression analyses showed that the total and reduced glutathione were significantly negatively associated or correlated with P450 activities, especially at the larval stage, while the association with GST and α & β-esterases were very low or not highly significant (Fig. 6.3 and 6.5). However, oxidized glutathione (GSSG) produced highly statistically significant positive associations with the activities of these enzymes ($p<0.05$) across the three life stages studied (Fig. 6.7). Also, a model deduced from factors and redundancy analysis prominently selected PCs correlating with GSSG across the three life stages, together with life stage-specific total and reduced glutathione as producing the most singular combined effect on the activities of the detoxification enzymes. Thus, as in the case of the detoxification enzymes, higher levels of GSSG was recorded in study zones A and C, which also recorded higher

levels of environmental chemical factors compared to zone B. The increased consumption of reduced glutathione (GSH) lead to the build up of higher levels of GSSG in these two zones (A & C) which then correlated positively with activities of detoxification enzymes. Therefore, it could be argued that while significant induction in the synthesis of glutathione may not have occurred in the *An. gambiae* sampled across the three zones, there was however a significant increase in utilization of GSH in mosquito samples collected from breeding sites where higher levels of environmental xenobiotics were recorded.

Hence, since GSH is an integral part of the overall detoxification process; acting directly to conjugate xenobiotics and indirectly as substrate to GST, the levels of the resultant GSSG correlated significantly with the activities of the detoxification enzymes. The positive association of GSSG with GST was obviously due to the role of GSH as a substrate for GST in the metabolism of xenobiotics, while the association with P450 and α & β-esterases could be due to their role in the further metabolism of GSH-conjugated metabolites (Lin and Yang, 2007). Finally, the increased accumulation of GSSG as indicated by the lower GSH/GSSG ratios in zone A and C, as compared to zone B (Tables 6.1, 6.2 & 6.3), could be due to the increased oxidative stress as a result of the higher xenobiotic overload. The increased oxidative stress was evidenced by the significantly higher levels of environmental chemical factors recorded in these zones compared to zone B. Also, these two zones recorded higher activities of the detoxification enzymes, hence the significant positive associations with GSSG. Observations from previous studies (Araujo *et al.*, 2008; Stephensen *et al.*, 2002) have established increase in oxidative stress induced by xenobiotic overload as a source of generation and accumulation of GSSG, leading to lower GSH/GSSG ratio in various organisms.

Distribution and changes in the levels of various forms of glutathione and their relationship with the activities of detoxification enzymes has not been largely investigated in insects.

However, findings from previous studies (Lin and Yang, 2007; Evelo *et al.*, 1993; Lee *et al.*, 1989; Virginia *et al.*, 2000; Aouacheri *et al.*, 2003) involving other organisms showed that changes in the levels of glutathione and activities of various detoxification enzymes do occur in response to different conditions of oxidative stress.

In this study, a low to moderate association was established between levels of total and reduced glutathione and activities of detoxification enzymes while a highly significant positive correlation was demonstrated between oxidized glutathione and activities of the three major detoxification enzymes (P450, GST and α & β-esterases) in *An. gambiae*. In literature as outline above, contradictory observations were reported regarding the levels of various forms of glutathione and their association with detoxification enzymes under conditions of oxidative stress in both plants and animals. In most instances (Chen *et al.*, 2004), the levels of total and reduced glutathione may increase, reduce or may not change significantly under conditions of oxidative stress. However, levels of oxidized glutathione and the ratio between oxidized and reduced forms of glutathione is usually used as the more accurate indicator of the redox state of a cell and in most organisms, this ratio defines the degree of oxidative stress induced either by xenobiotic overload or free radical formation, especially in instances where no apparent and significant induction in the synthesis of glutathione occurred (Chen *et al.*, 2004; Araujo *et al.*, 2008). Hence, finding from this present study appeared to be consistent with these observations. Despite the low significance in the levels of total and reduced glutathione across the three study zones, the highly statistically significant differences in the levels of GSSG across these zones could be used to explain the varying oxidative stress conditions as indicated by the significant changes in the activities of the detoxification enzymes across these zones.

Furthermore, another line of argument in support of the observed low changes in the levels of the total and reduced glutathione across the three study zones despite significant differences

in the levels of environmental chemical factors is that glutathione is constitutively synthesized and abundantly available in all organisms. Under normal physiological conditions, its concentration in higher organisms such as mammals ranges between 2-10 mM (Pastore *et al.*, 2003). Synthesis of glutathione can however be induced under increased conditions of oxidative stress (Pastore *et al.*, 2003). Thus, it is possible that the levels of reduced glutathione recorded in this study represent the normal threshold levels in *An. gambiae*. This means that despite the observed inductive effect of the chemical environmental factors on the activities of the detoxification enzymes studied (Chapter 5), these levels of glutathione was sufficient for its role in the overall detoxification process. Hence, since levels, availability and activities of glutathione has been shown to respond to changes in oxidative stress induce by xenobiotic overload (Lu, 2009), the sources of oxidative stress in our samples of *An. gambiae* (i.e. the chemical environmental factors) may not be significantly sufficient enough to cause the induction of glutathione synthesis above the normal threshold levels even though it was sufficient to induce increased activities of the detoxification enzymes.

6.5 Conclusion

This study was carried out to investigate the distribution of the various forms of glutathione and their relationship with activities of detoxification enzymes in *An. gambiae* mosquitoes under varying degree of oxidative stress induced by environmental xenobiotics. The results and observations recorded suggest that while significant induction in synthesis of glutathione may not have occurred, there was however an approximately 2-fold increase in the utilization of glutathione which corresponds with the degree or levels of xenobiotic concentrations across the three zones studied. The relationship between oxidized glutathione (GSSG) and the activities of the three major detoxification enzymes (i.e. P450, GST and α & β-esterases) in *An. gambiae* demonstrated in this study suggest a close coordinated relationship between

activities of glutathione and these detoxification enzymes in xenobiotic metabolism. Since many of the environmental chemical factors investigated in this study have been established to share similar structures and activity relationship with many of the chemical insecticides used in malaria vector control (Chapter 3) , their routes of metabolism and hence, the response they elicited in mosquito detoxification process could also be similar. Therefore, observations from this study and inferences from previous findings suggest a close coordination and relationship in the synthesis, activities and regulation of both glutathione and the detoxification enzymes. This could be harnessed in designing a novel strategy for the regulation of synthesis and activities of these enzymes, as a tool for the management of insecticides resistance.

CHAPTER SEVEN

7.0 Concluding Remarks, Conclusion and Recommendations

7.1 Concluding Remarks

The results and observations recorded in this PhD research confirmed or proved, within limits of all the experimental conditions, the aim or the working hypothesis of this study- that environmental xenobiotics present in mosquito breeding ecologies could produce a selection pressure for the development of insecticide resistance in *An. gambiae*. *Anopheles gambiae* mosquitoes were sampled form different breeding ecologies defined by marked differences in environmental xenobiotic concentrations in Nigeria. The types and levels or concentrations of these environmental xenobiotics/factors were a function of the different human related activities taking place within and/or around the mosquito breeding sites. The *An. gambiae* breeding sites productivity, measured as larval density, was significantly associated with the degree of xenobiotic concentrations. This means that while *An. gambiae* may still prefer cleaner, clearer and uncontaminated breeding water, as signified by the higher larval density densities recorded from those breeding sites with very low levels of environmental chemical factors, large amount of larvae were nonetheless found to be thriving in breeding ecologies with comparably very high levels of these xenobiotics. This suggests a gradual emergence of tolerance by *An. gambiae* to the presence of high levels of environmental contaminants in their breeding ecologies.

Detoxification enzymes analysis carried out on the three life stages of the *An. gambiae* emerging from the aforementioned breeding ecologies confirmed the display of tolerance to high levels of these environmental chemical parameters through biochemical detoxification mechanism. P450 activities was 2 and 4-fold lower in zone A & B compared to zone C while GST and CEs activities were 9-fold higer in zone A compared to zones B & C. Furthermore,

statistically significant associations ($p<0.05$) were established between these detoxification enzymes and the environmental chemical factors; suggesting an induction in synthesis and/or activities of these enzymes in response to the xenobiotic overload. This is a common defence mechanism that allows most organisms to adapt to or tolerate changing environmental physical and chemical stress conditions in their habitats. In addition, the role of glutathione in the overall detoxification process was assessed by determining the distribution of the various forms of glutathione (total, reduced and oxidized glutathione) in the three life stages of the *An. gambiae* collected from the three study zones. The results suggests that while significant induction in the synthesis of glutathione may not have occurred in response to the varying degree of xenobiotic concentrations across the three studied zones, there was however, a significant increase in utilization of glutathione. This increased utilization of glutathione signified by the significant differences in the levels of oxidized glutathione (GSSG), was significantly associated with the activities of the three major detoxification enzymes studied. This suggests a coordinated role for both glutathione and the activities of not only the glutathione dependent enzyme (i. e. GST), but also the P450 and CEs studied. Therefore, these findings supported by inferences from previous studies could inform a novel or an alternative regulatory approach for these enzymes in the management of insecticides resistance in mosquito.

The significance of the overall findings in this PhD study to the development or emergence of insecticide resistance after exposure of *An. gambiae* to different classes of insecticides cannot be underestimated. Firstly, chemical categorization studies showed that some of the investigated environmental chemical factors share similar structures and activity relationship with most of the chemical synthetic insecticides used in malaria vector control. Responses from farmers interviewed in farmlands around the intensive agricultural areas, who confirmed the use of different types of agro-pesticides and fertilizers on their farms, (study zone A) lend

credence to this observation. These agro-pesticides, all of which belong to the known chemical classes of the synthetic insecticides used in malaria vector control, are washed by rain form farmlands where they are applied into any surrounding mosquito breeding sites. Hence, chemical species/factors such as sulphates, phosphates and carbon content which forms some of the integral chemical structures of most insecticides or their break down products, were detected in very high levels in this zone compared to the domestic or residential areas (study zone B). In addition, nitrite and nitrate ions from fertilizer application were also found in significantly higher levels in study zone A. Finally carbon content and oil and grease were detected in higher levels in study zone C, which is defined by commercial activities involving the sale, processing, use and /or discharge of petrochemical and other hydrocarbon products into the environments. The presence of high levels of carbon content and oil and grease from breeding sites located in this zone suggests that these products are also carried by rain water into any surrounding mosquito breeding sites. Thus, study zones A and C, defined by intensive agriculture and hydrocarbon related commercial activities respectively, account for a significantly higher concentrations of the environmental xenobiotics examined in this study compared to zone B, where these agricultural or commercial activities were mostly non existence.

Furthermore, most environmental xenobiotics including insecticides follow the same major detoxification process in most organisms. This process involves three major detoxification pathways usually referred to as the phase I, II, and III detoxification pathways. These three phases of detoxification employs the use of –in addition to other molecules and co-factors- the three major detoxification enzyme families namely; P450s, GSTs and CEs. A vast network of isoenzyme subfamilies allows these three families of detoxification enzymes to metabolise almost any xenobiotic compounds in most organisms. As described in various previous studies, changes in synthesis and activities of these enzymes is one major

mechanism employed by various disease vectors (*An. gambiae* inclusive) to reduce the effectiveness of public health insecticides used for their control, thus giving rise to insecticides resistance in these vectors. Upregulation of synthesis and activities of these enzymes has been used by *An. gambiae* to reduce the effectiveness of almost all the public health insecticides employed in malaria control. With higher enzyme activities in resistant strains of *An. gambiae*, most of the insecticide molecules would be metabolised before they reach their site of actions (sodium ion-gated channel in the nerve synapses or Acetylcholinesterase receptors in the nerve shealths). Therefore, factors which could induce the synthesis and activities of these enzymes in *An. gambiae* prior to insecticide exposure could act to select them for development of metabolic resistance after exposure. This means that *An. gambiae* strains displaying increased activities of these enzymes in response to high levels of xenobiotic exposure are more likely to develop metabolic resistance to insecticides upon exposure.

Finally, the problem of insecticide resistance is the major obstacle to malaria control and eradication programmes, leading to continuous deaths of millions especially children, in malaria endemic sub-Saharan Africa. Different approaches have been employed to tackle this problem with no concrete pathway yet for its elimination. However, inferences from previous studies and advances in the understanding of the underlying mechanisms of insecticides resistance in various vectors have indicated that management of insecticides resistance rather than elimination of insecticides resistance is the most viable and effective strategy for malaria vector control. And since changes in the synthesis and activities of these detoxification enzymes constitute one major mechanism of insecticide resistance, studies targeting the regulation and control of their synthesis and activities are necessary for any effective insecticide management approach to malaria vector control. Consequently, any environmental factors that could exacts selection pressure on malaria vectors leading to

development of resistance after insecticide exposure has to be taken into cognisance for any insecticide management programmes to be effective, hence this study.

7.2 Conclusion

The findings from this PhD study has demonstrated that various environmental xenobiotic compounds present in *An. gambiae* breeding ecologies- in varying levels or concentrations- could exacts a powerful selection pressure on *An. gambiae* for development of metabolic insecticides resistance. Most of the previous studies on resistance mechanisms and management have concentrated largely at the level of post-insecticide exposure. However, findings from this study and inferences from few related previous studies suggests that xenobiotic characteristics of some *An. gambiae* breeding ecologies could be acting to prime *Anopheles* mosquito for resistance to insecticides even before exposure. Thus *An. gambiae* mosquito emerging from certain ecologies could have developed appropriate enzymatic machinery or arsenal that would equip them with the adaptive or acquired detoxification capacity to develop rapid tolerance to any insecticides used for their control.

7.3 Recommendations

This study was conducted at pre-insecticide exposure level, employing some of the mechanisms for resistance detection and management in *An. gambiae*. This is to establish the activities of the aforementioned environmental xenobiotic factors as agents that could produce selection pressure towards resistance development following insecticides exposure. The following recommendations are hereby proposed:

1). Further studies at post-insecticides level are needed to complement the findings in this study. Specifically, studies involving insecticides bioassay, employing different classes of insecticides, on *An. gambiae* sampled from different breeding sites with different xenobiotic

characteristics-such as the ones studied here- should be carried to examine the response of *An. gambiae* from such ecologies to the different classes of insecticides, and thus examine the relationship or associations between such responses, if any, and the environmental xenobiotic conditions.

2). Further studies on any endogenous compounds or molecules that are known to interact with the detoxification enzymes during xenobiotic metabolic process-such as glutathione studied here- are necessary in order to explore the possibility of using such compounds in the control and regulation of synthesis and activities of the detoxification enzymes. Such studies would provide more understanding towards the effective use of the regulation of these enzymes as tool for insecticide resistance management.

3). Specifically, further studies on the synthesis, activities and regulation of the various forms of glutathione and their relationship with the detoxification enzymes are necessary. Such studies could provide a novel understanding and approach that could be employed in the regulation of synthesis and activities of the detoxification enzymes in *An. gambiae*.

4). There is need also to carry out investigation on the activities of any other environmental xenobiotic compounds- other than the ones studied here- that may be present in *An. gambiae* breeding ecologies with a view to examining their impact, if any, on the growth, development and behaviour of the mosquitoes emerging from such ecologies, especially towards any vector control initiatives.

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APPENDIX

Appendix A: Further Results

Appendix A1 Further Results on Glutathione Studies

Table 1 Combined zone-wise levels (nmol/mgprotein) of the three forms of glutathione (total, reduced and oxidized) across the three life stages of An. gambiae sampled form three different breeding ecologies (study zones) in Nigeria.

Life Stages of *An. gambiae*	Total GSH	GSH	GSSG	Study Zone[b]
Larvae	68.97±2.86[a]	59.44±2.46	9.65±0.40	A
	68.67±3.14	61.94±2.84	6.72±0.29	B
	61.65±3.18	51.78±2.67	9.86±0.51	C
Pupae	57.39±2.58	44.77±2.02	12.63±0.57	A
	59.10±2.43	50.83±2.09	8.28±0.34	B
	49.07±2.87	41.21±2.41	7.85±0.46	C
Adult	50.38±2.42	36.70±2.26	13.67±0.58	A
	48.45±1.46	42.61±1.31	5.86±0.18	B
	41.12±2.25	33.75±1.49	7.37±1.01	C

[a]Mean ± S. D for three determinations.

[b]Zone: A, intensive agriculture; B, residential/domestic; and C, petrochemical breeding ecologies

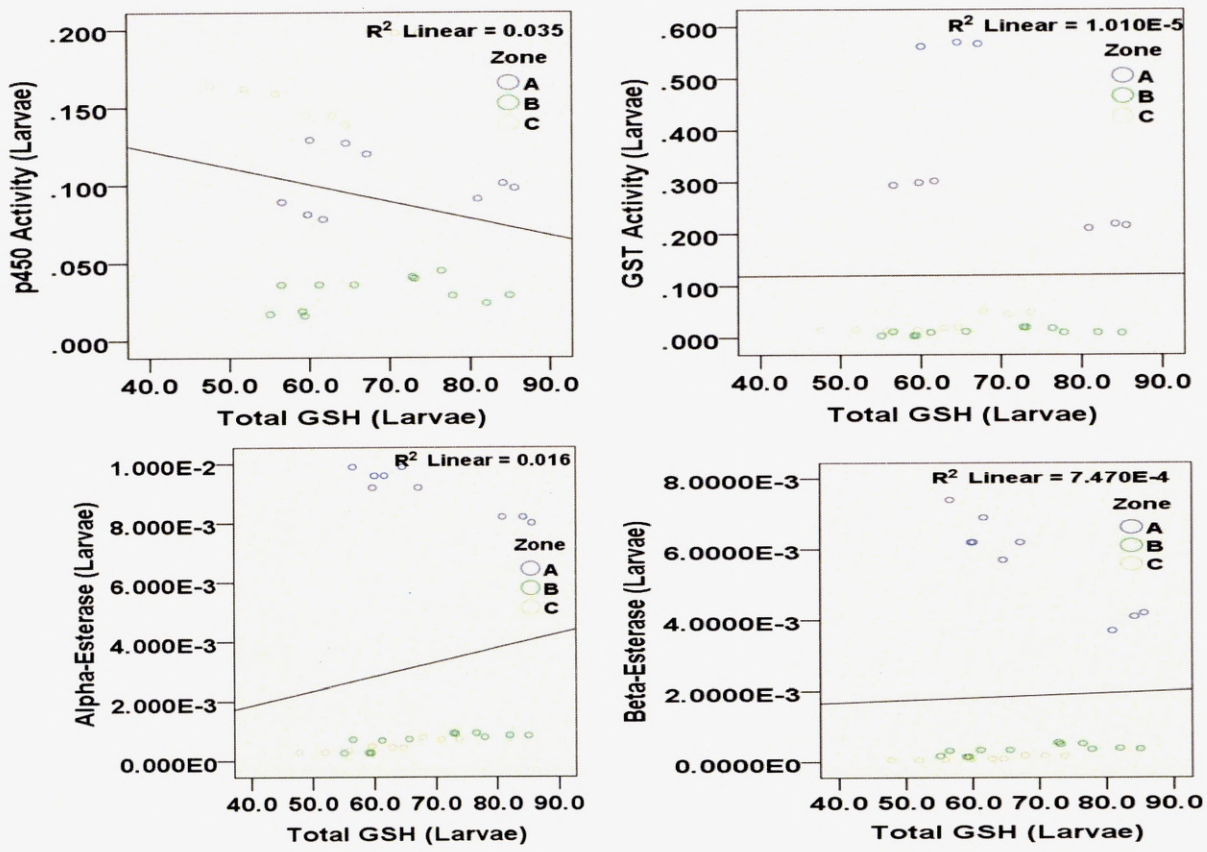

Fig.1 Associations/correlations between total glutathione (tGSH) and activities of p450, GST and α & β-esterases at the larval stage of *Anopheles gambiae*

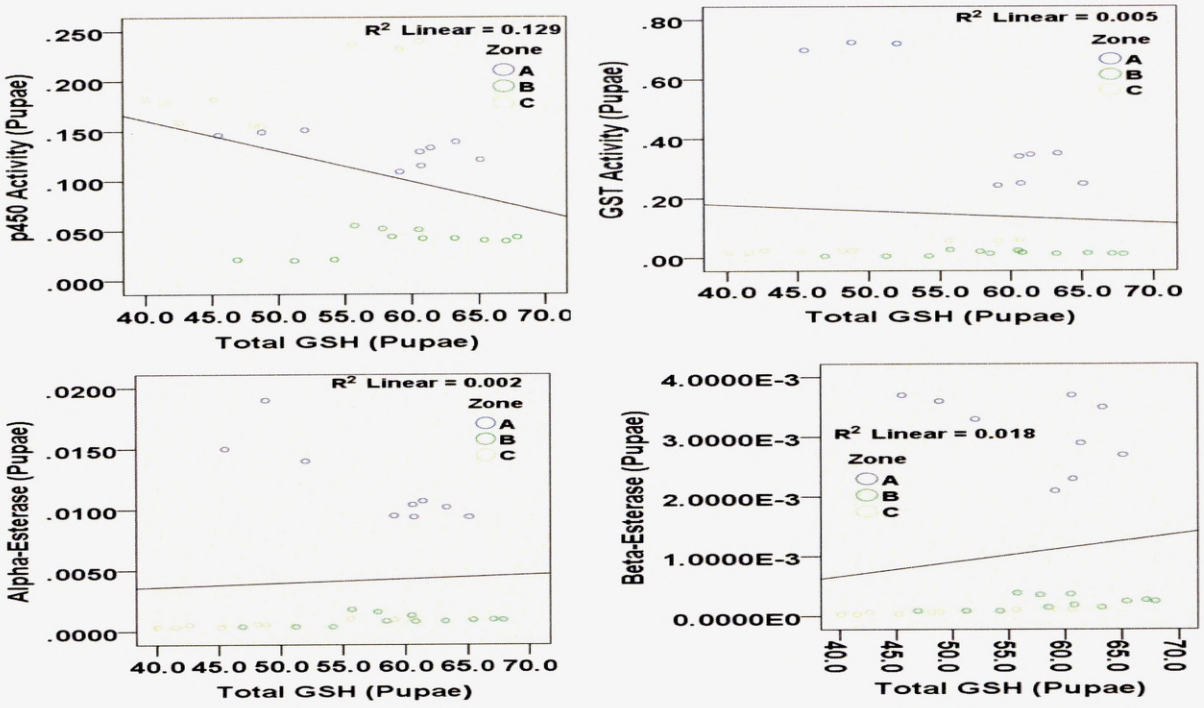

Fig.2 Associations/correlations between total glutathione (tGSH) and activities of p450, GST and α & β-esterases at the pupal stage of *Anopheles gambiae*

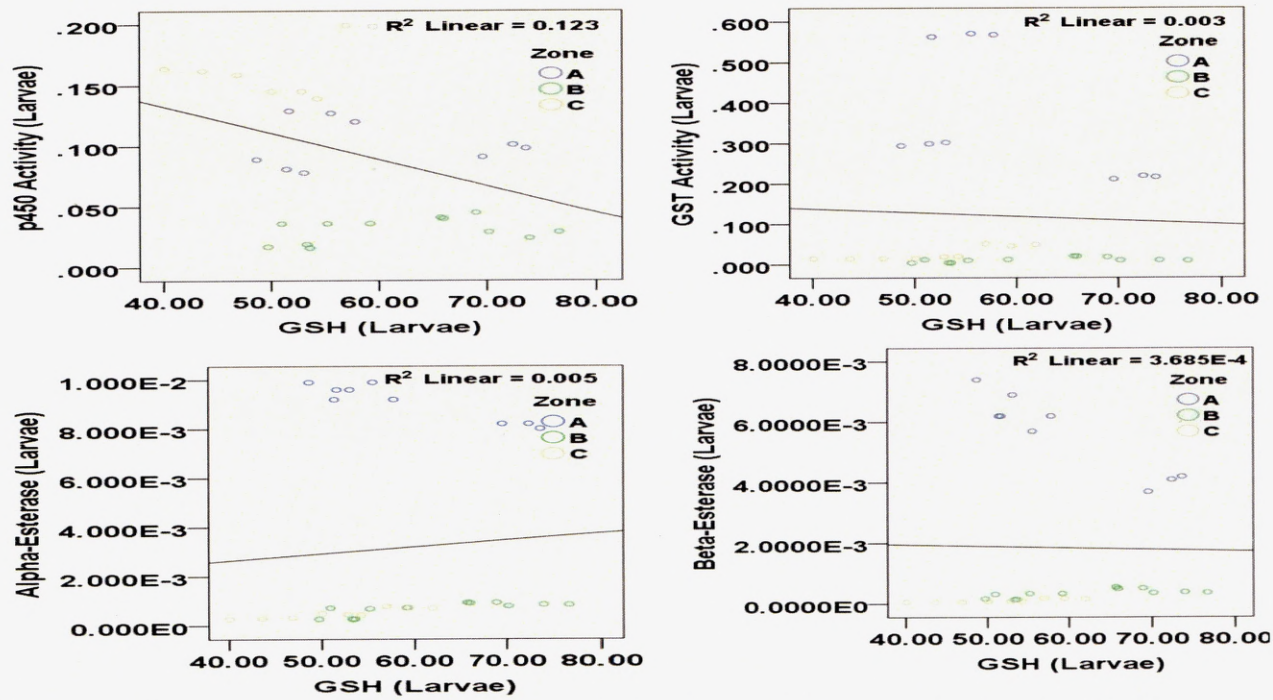

Fig.3 Associations/correlations between total glutathione (tGSH) and activities of p450, GST and α & β-esterases at the larval stage of *Anopheles gambiae*

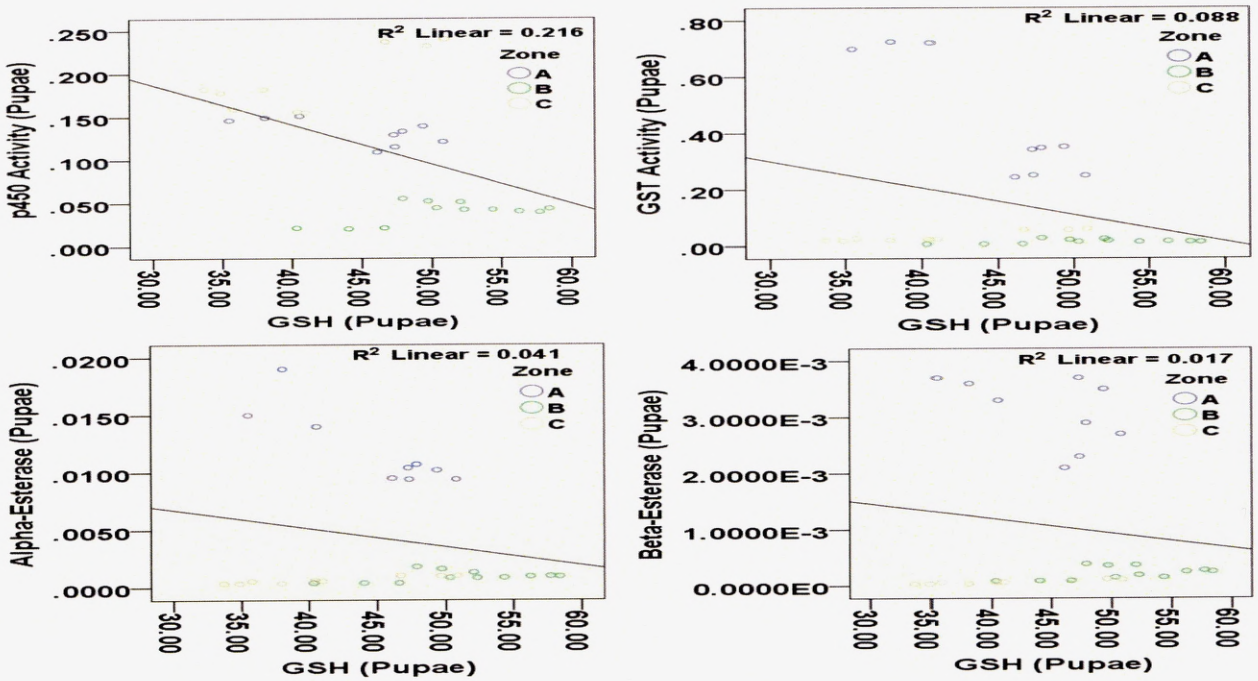

Fig.4 Associations/correlations between total glutathione (tGSH) and activities of p450, GST and α & β-esterases at the pupal stage of *Anopheles gambiae*

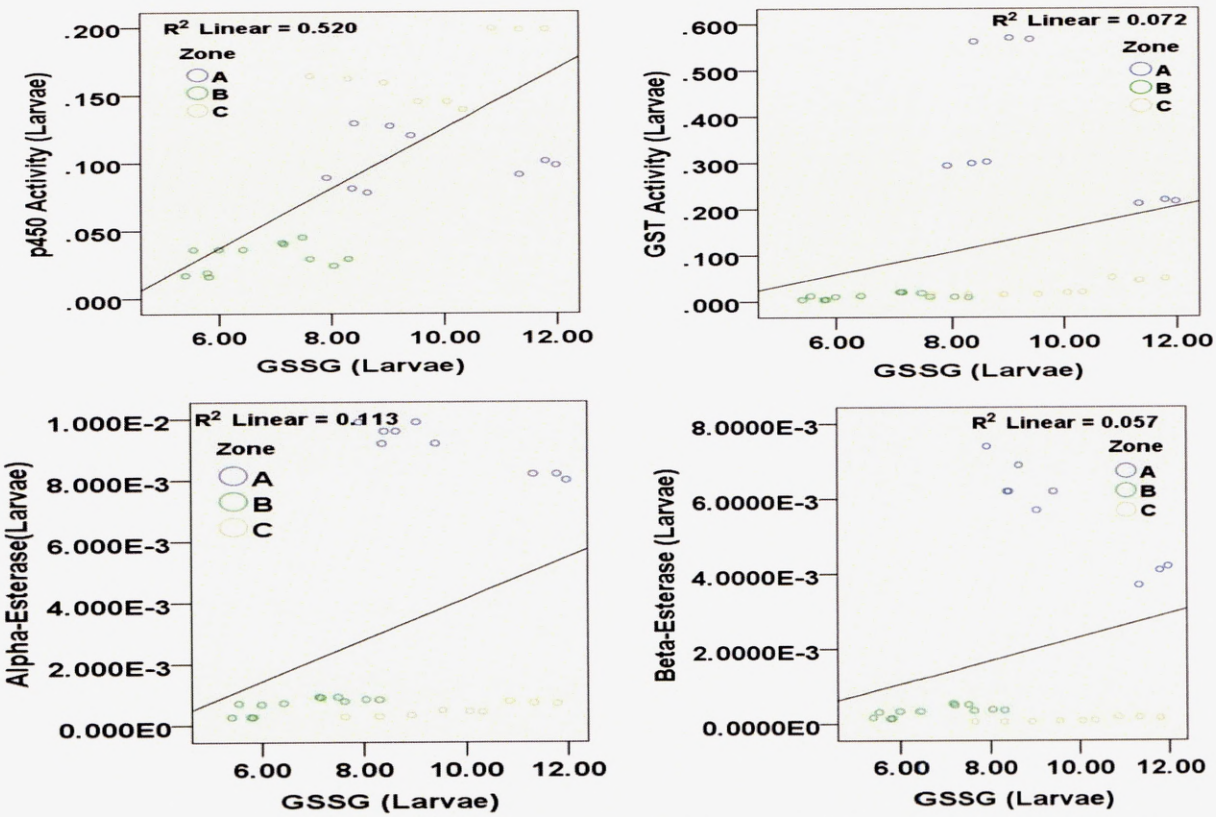

Fig.5 Associations/correlations between total glutathione (tGSH) and activities of p450, GST and α & β-esterases at the larval stage of *Anopheles gambiae*

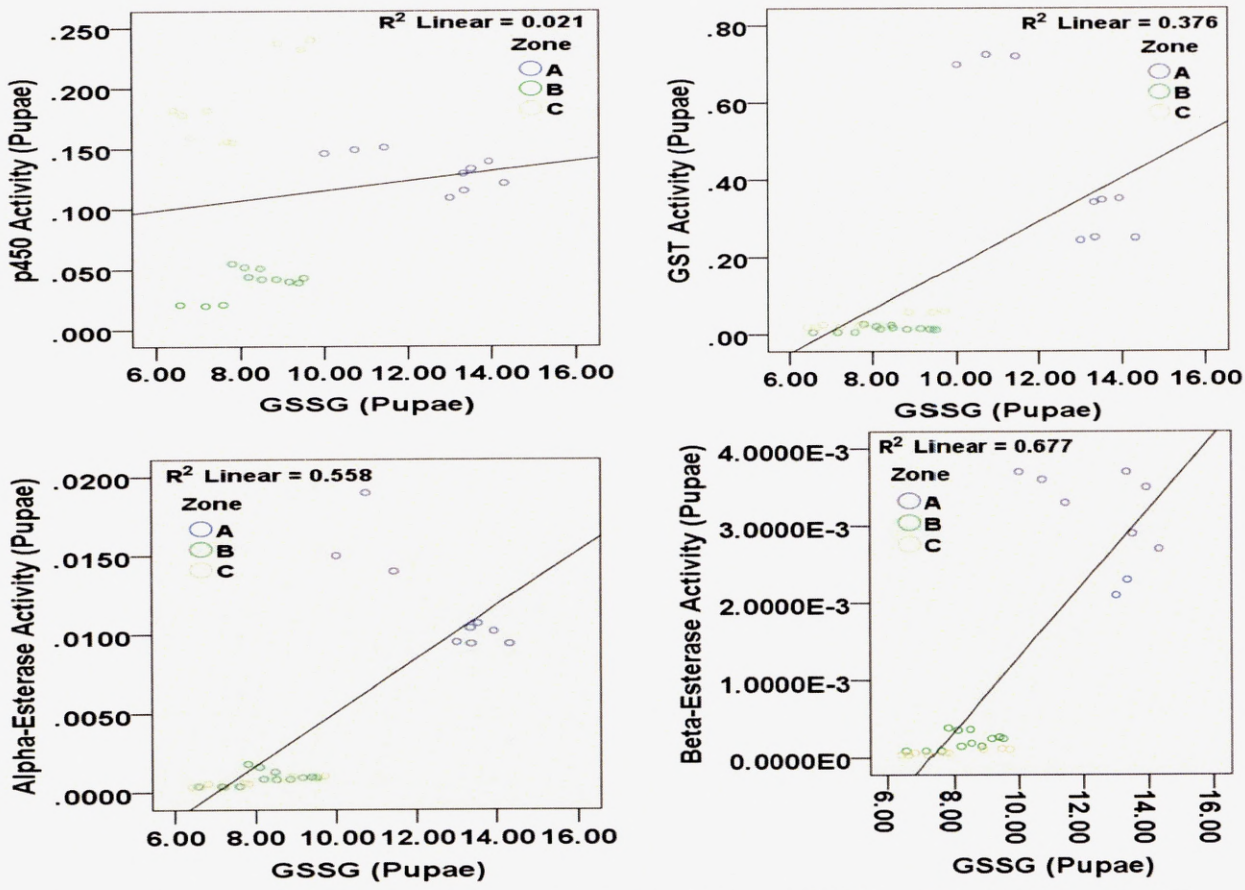

Fig.6 Associations/correlations between total glutathione (tGSH) and activities of p450, GST and α & β-esterases at the pupal stage of *Anopheles gambiae*

Appendix A2: Further Results on Factor and Redundancy Analysis

Table 1 Total components/factor variance for factor analysis of the environmental physico-chemical parameters

Total Variance Explained

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	5.948	45.751	45.751	5.948	45.751	45.751	5.036	38.740	38.740
2	2.875	22.116	67.867	2.875	22.116	67.867	2.857	21.974	60.714
3	2.005	15.423	83.290	2.005	15.423	83.290	1.129	8.687	69.401
4	.935	7.194	90.484	.935	7.194	90.484	1.077	8.288	77.689
5	.515	3.958	94.442	.515	3.958	94.442	1.073	8.250	85.940
6	.375	2.882	97.323	.375	2.882	97.323	.874	6.726	92.666
7	.206	1.581	98.905	.206	1.581	98.905	.778	5.983	98.649
8	.114	.879	99.783	.114	.879	99.783	.137	1.056	99.705
9	.016	.121	99.904	.016	.121	99.904	.021	.162	99.867
10	.009	.069	99.974	.009	.069	99.974	.011	.081	99.948
11	.003	.021	99.995	.003	.021	99.995	.006	.046	99.994
12	.000	.004	99.998	.000	.004	99.998	.001	.004	99.998
13	.000	.002	100.000	.000	.002	100.000	.000	.002	100.000

As showed in this table, components 1-8 explained the cumulative percentage variance in the data and were thus selected as the only factors correlating with all the physico-chemical environmental variables.

Table 2 Components or factors correlating with each of the physico-chemical environmental variables

Rotated Component Matrix

	Component												
	1	2	3	4	5	6	7	8	9	10	11	12	13
pH	.229	.506	-.060	.200	-.095	-.134	.788	-.011	.000	-.002	.001	.000	.000
Temp	.132	.206	.267	.886	.150	.200	.146	.015	.001	.000	.000	.000	.000
Conduct.	-.218	.058	.938	.222	.020	.133	-.039	.011	-.001	.000	.001	.000	.000
D.O	-.434	-.068	.202	.262	.147	.813	-.123	.015	.001	-.001	.000	.000	.000
B.O.D	.255	-.813	.213	.206	.130	.209	-.191	.298	.006	-.001	-.001	-.001	.000
TDS	.916	-.323	-.023	.004	-.148	-.106	-.115	-.023	-.019	.091	-.007	.000	.000
Transparency	-.008	-.087	.020	.109	.985	.086	-.052	.006	.000	-.001	.000	.000	.000
Sulpahtes	.948	-.113	.021	.022	-.017	-.222	.160	-.077	-.040	-.025	.064	.000	-.002
Phosphates	.964	-.119	-.138	.103	-.007	-.033	.062	.124	.065	-.026	-.039	.002	-.011
Nitrites	.955	-.166	-.127	.005	.081	-.135	.070	-.075	-.094	.002	.000	-.001	.003
Nitrates	.964	-.169	-.142	.022	.048	-.039	.080	.066	.078	-.025	-.014	-.002	.010
Carbon content	-.198	.945	.103	.170	-.038	.074	.142	.042	.002	-.011	.008	-.015	.001
Oil & Grease	-.342	.895	.144	.200	-.010	.012	.075	.119	.003	.009	-.010	.018	-.001

Components of factors 1-8 were selected because they explained all the variability in the data.

Table 3 Total components/factor variance for factor analysis of the detoxification enzyme variables

Total Variance Explained

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	8.577	71.474	71.474	8.577	71.474	71.474	8.450	70.414	70.414
2	2.924	24.363	95.838	2.924	24.363	95.838	2.951	24.592	95.006
3	.390	3.247	99.084	.390	3.247	99.084	.482	4.019	99.026
4	.068	.567	99.651	.068	.567	99.651	.065	.538	99.564
5	.017	.143	99.794	.017	.143	99.794	.018	.149	99.713
6	.010	.087	99.881	.010	.087	99.881	.017	.143	99.856
7	.010	.082	99.963	.010	.082	99.963	.012	.101	99.957
8	.002	.017	99.980	.002	.017	99.980	.003	.021	99.978
9	.002	.014	99.994	.002	.014	99.994	.002	.016	99.994
10	.001	.004	99.998	.001	.004	99.998	.001	.005	99.998
11	.000	.001	99.999	.000	.001	99.999	.000	.001	99.999
12	6.070E-005	.001	100.000	6.070E-005	.001	100.000	.000	.001	100.000

As showed in this table, components 1-3 explained the cumulative percentage variance in the data and were thus selected as the only factors correlating with all the detoxification enzyme variables

Table 4 Components or factors correlating with each of the detoxification enzyme variables

Rotated Component Matrix

	Component											
	1	2	3	4	5	6	7	8	9	10	11	12
p450(L)	.072	.992	.061	-.031	-.018	.008	.073	-.021	.000	.000	.000	.000
p450(P)	.179	.981	-.014	.050	.021	-.005	.003	.040	.002	-.001	.000	.000
p450(A)	-.157	.984	.005	-.021	-.002	-.002	-.078	-.018	-.002	.000	.000	.000
GST(L)	.932	.106	.347	-.003	.012	.004	-.003	-.002	.000	.001	.001	.008
GST (P)	.915	.107	.389	-.001	.001	.009	.003	.002	.000	.000	-.007	-.005
GST(A)	.924	.105	.367	-.012	-.002	.004	.001	-.005	.001	-.001	.008	.000
Alpha-Esterase(L)	.996	.015	-.058	-.041	-.033	-.003	.018	.007	-.004	-.019	.000	.000
Alpha-Esterase(P)	.972	.034	.182	-.083	-.009	.118	.003	-.001	-.001	.000	.000	.000
Alpha-Esterase(A)	.979	.012	-.151	-.119	-.052	-.021	.018	.005	-.006	.012	-.001	-.001
Beta-Esterase(L)	.989	-.009	-.044	.131	.002	-.034	.011	.005	.035	.002	.000	.000
Beta-Esterase(P)	.993	-.007	.018	-.001	.112	-.012	-.003	.002	.000	.001	.000	.000
Beta-Esterase(A)	.983	-.009	-.102	.144	-.022	-.035	.000	.005	-.025	.006	-.001	-.001

Components of factors 1-3 were selected because they explained all the variability in the data.

Table 5 Total components/factor variance for factor analysis of the detoxification enzyme variables

Total Variance Explained

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	4.881	54.228	54.228	4.881	54.228	54.228	2.207	24.521	24.521
2	2.313	25.702	79.930	2.313	25.702	79.930	2.181	24.237	48.758
3	.988	10.976	90.905	.988	10.976	90.905	1.991	22.121	70.879
4	.606	6.738	97.643	.606	6.738	97.643	1.607	17.859	88.737
5	.199	2.208	99.852	.199	2.208	99.852	.998	11.084	99.821
6	.013	.148	100.000	.013	.148	100.000	.016	.179	100.000
7	3.537E-016	3.930E-015	100.000	3.537E-016	3.930E-015	100.000	3.249E-016	3.610E-015	100.000
8	-4.834E-017	-5.371E-016	100.000	4.834E-017	5.371E-016	100.000	2.420E-016	2.689E-015	100.000
9	-2.000E-016	-2.222E-015	100.000	2.000E-016	2.222E-015	100.000	1.224E-016	1.360E-015	100.000

As showed in this table, components 1-5 explained the cumulative percentage variance in the data and were thus selected as the only factors correlating with all the glutathione variables.

Table 6 Components or factors correlating with each of the glutathione variables

Rotated Component Matrix

	Component								
	1	2	3	4	5	6	7	8	9
tGSH_2(L)	.313	.145	.849	.282	.284	.002	.000	.000	.000
tGSH_2(P)	.880	.246	.304	.265	-.035	.021	.000	.000	.000
tGSH_2(A)	.294	.438	.350	.773	.010	-.027	.000	.000	.000
GSH (L)	.359	.084	.862	.325	.125	.002	.000	.000	.000
GSH (P)	.900	-.036	.307	.293	-.089	-.007	.000	.000	.000
GSH (A)	.412	-.152	.361	.807	-.160	.023	.000	.000	.000
GSSG (L)	-.102	.358	.263	-.102	.884	.000	.000	.000	.000
GSSG (P)	.334	.918	.126	.038	.140	.090	.000	.000	.000
GSSG (A)	-.128	.951	.042	.078	.255	-.079	.000	.000	.000

Components of factors 1-3 were selected because they explained all the variability in the data.

Appendix B: Reagents and Reagent Preparations

1). Reagents Preparation for BOD Test

Phosphate buffer solution: 8.5g KH_2PO_4, 21.75g K_2HPO_4, 33.40g$Na_2PO_4.7H_2O$, and 1.7g NH_4Cl were dissolve in 500ml of distilled, the pH was adjusted to 7.2 and the volume made up to 1L.

Magnesium Sulphate Solution: 22.5g of $MgSO_4$ was dissolved in distilled water and the volume made up to 1L.

Calcium Chloride Solution: 27.5g of anhydrous $CaCl_2$ was dissolved in 1L of distilled water.

Ferric Chloride Solution: 0.25g $FeCl_3.6H_2O$ was dissolved in 1L of distilled water.

2). Reagents Preparation for Sulphate ion Determination.

Conditioning reagent: 50ml glycerol was mixed with a solution containing 30ml concentrated HCl, 300ml distilled water, 100ml of 95% isopropyl alcohol, and 75g NaCl.

Stock Sulphate solution: 100mg/ml stock sulphate solution was prepared by dissolving 10g of anhydrous sodium sulphate (Na_2SO_4) in 50ml of distilled water, the solution was shaken and the volume made up to 100ml. 100ml each of 5mg/ml, 10mg/ml, 15mg/ml, 20mg/ml and 25mg/ml of sulphate standards solution were then prepared from the 100mg/ml stock solution using appropriate dilutions.

3). Reagents Preparation for Phosphate ion determination:

Ammonium molybdate solution: Ammonium molybdate solution was prepared by dissolving 2.5g of ammonium molybdate in 17.5ml of distilled water followed by 28ml of Conc. H_2SO_4. The solution was made up to 100ml by distilled water.

Stock phosphate solution: 100ml of 100mg/ml stock phosphate solution was prepared by dissolving 10g of Potassium dihydrogen phosphate (KH_2PO_4), in 100ml of distilled water. Phosphate standards ranges between 5-25mg/ml were prepared from the stock solution using appropriate dilutions.

Stannous chloride solution: Stannous chloride solution was prepared by dissolving 2.5g of fresh $SnCl_2$ in 100ml of glycerol. The solution was then heated in a water bath and stirred with a glass rod to hasten dissolution.

4). Reagents Preparation for nitrite ion determination:

Colour reagent: The colour reagent was prepared by mixing 800ml of distilled water with 100ml of 85% phosphoric acid and 10g sulphanilamide. After mixing, the volume was made up to 1000ml.

Stock and standard nitrite solutions: Stock solution of nitrite ion was prepared by dissolving 0.4926g of anhydrous sodium nitrite ($NaNo_2$) previously dried for 24 hours in a dessicator, in distilled and the volume made up to 1L. Working solution was prepared by dissolving 10ml of the stock to 1L by with distilled water. Standard nitrite solutions of 0.02-0.30mg/L were prepared from the working solution using appropriate dilutions.

5). Reagents Preparation for nitrate ion determination:

Phenol disulfonic acid (PDA) reagent: 25g of white phenol was dissolved in 150ml of conc. H_2SO_4 foolowed by the addition of 75ml of fuming sulphuric acid. The solution was stirred and heated for 2 hours on water bath.

Silver sulfate solution: 4.40g of Ag_2SO_4 was dissolved in 1000ml of distilled water. (1ml removes 1mg of chloride).

Potasium hydroxide solution: 12N KOH solution was prepared by dissolving 673g of KOH salt in 1L of distilled water.

Aluminium hydroxide solution: 125g potash alum was dissolved in 1000ml of distilled water, heated to 60°C and 60ml of conc. ammonium hydroxide was added. The solution was allowed to stand for 1 hour, the supernatant was decanted and the precipitate washed few times to remove residual chlorine or nitrite.

Stock and standard nitrate solution: Stock nitrate solution was prepared by dissolving 721.8mg of anhydrous potassium nitrate in 1000ml of distilled water; 1ml = 100µgNO_3^-. Standard nitrate solutions were prepared by first evaporating 50ml of the stock solution to dryness on water bath, redissolved inn 2ml of PDA reagent, and diluted to 500ml; 1ml= 10µgNO_3^-.

EDTA solution: 50g of EDTA was first dissolved in 20ml of distilled water. 60ml of NH_4OH was then added and mixed.

6). Reagents Preparation for detoxification enzyme assays:

Sodium phosphate buffer solutions: One litre of 1M stock sodium phosphate buffer was prepared by first dissolving 142.g of Na_2HPO_4 (Dibasic) in 1L of distilled water and 142.g NaH_2PO_4 (Monobasic) also in 1L of distilled water. The two solutions were then mixed together in the appropriate volumes to produce 1L of sodium phosphate buffer pH 7.0. Then, 0.02M and 0.1M solutions were prepared from the stock 1M solution using appropriate dilutions.

Potassium buffer solutions: One litre of stock potassium buffer was prepared by first dissolving 174g of K_2HPO_4 (dibasic) in 1L of distilled water and 138g of KH_2PO_4 (monobasic) also in 1L of distilled water. The two solutions were mixed in appropriate

volumes to produce 1L of potassium phosphate buffer pH 7.0. 0.625M solution was then prepared from the 1M stock solution using appropriate dilutions.

$3,3^1,5,5^1$-tetramethyl benzidine solution: 0.002mg/ml solution of $3,3^1,5,5^1$-tetramethyl benzidine was prepared by dissolving 0.01g of $3,3^1,5,5^1$-tetramethyl benzidine in 5ml of methanol.

Glutathione solution: 10mM glutathione solution was prepared by dissolving 0.0081g of reduced glutathione (GSH) in 2.5ml of 0.1M sodium phosphate buffer pH 6.5

Chlorodinitrobenzene (CDNB) solution: 63mM CDNB solution was prepared by dissolving 0.1278g of CDNB in 10ml of methanol.

Naphthyl acetate (NA) solution: 30mM 1-naphthyl acetate and 30mM 2-naphthly acetate solutions were prepared by dissolving 0.2793g of each of the 1 and 2-NA in 50ml of acetone.

Sodium lauryl sulphate solution (SDS): 5% SDS solution was prepared by dissolving 5g of SDS in 100ml of distilled water.

Stain solution: Stain solution for α and β-esterase activity assays was prepared by first dissolving 150mg of Fast blue B salt in 15ml of distilled water followed by addition of 35ml of 5% SDS

Appendix C: Selected Field Study Photos